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IN VITRO METABOLISM OF SUBSTITUTED STEROIDS BY RAT LIVER[1,2]

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ABSTRACT

The metabolism of 14 steroids with various structures has been investigated in liver slices. In all cases the rate of destruction was estimated by the Porter-Silber reaction to determine loss of the dihydroxyacetone sidechain; by the BTZ reaction to determine reducing power of sidechain without the 17-hydroxyl; by the absorption at 240 mμ to determine reduction in the Ring-A; and, by the Gornall reaction to determine alteration in reactivity to 2,4 dinitrophenylhydrozine. Substitution in Ring-A (1:2 ethylenic linkage, 2 methyl) did not change sidechain reaction, but depressed saturation of the Ring-A. Substituents in the 9 position had a similar effect on rate of metabolism. Natural steroids other than cortisone and hydrocortisone appeared to be inactivated much more readily than the substituted steroids. The activity of the substituted steroids cannot be explained solely on the basis of rate of metabolism.

IT IS well known that a slight alteration of the basic structure of the adrenal cortical steroids will increase activity of the molecule several times (1, 2). For example, introduction of a fluorine atom at position 9 increases the sodium retention 500 fold. Several rational theories may be proposed for the increased activity. One assumes that the liver is unable to metabolize the altered steroid, it remains in the blood and as a result has a higher activity. The present report deals with the ability of rat liver to metabolize substituted steroids, with results which bear directly on this question.

METHODS

Rats were decapitated, the liver was rapidly removed, cooled, and placed in a chilled dish. Slices were cut by the Deutsch method (3), 250 mg. were weighed on a torsion balance, and transferred to wide mouth bottles of 25 ml. capacity containing 3 ml. of

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[2] The steroids were obtained through the very kind efforts of Dr. Alpert, Merck & Co.; Dr. Byrne, Upjohn Co.; Dr. Gaunt, Ciba; Dr. Drill, G. D. Searle; and Dr. Bird, Sterling-Winthrop.

Kreb's-bicarbonate buffer (pH 7.4) which were placed in a Dubnoff Shaker for temperature equilibration at 38° C, after which 500 μg. of the free alcohol of the desired steroid in 0.2 ml. of ethanol[3] were added. Great care was taken to ensure that the steroid was in solution. The flasks were shaken at 120 RPM for one hour in an atmosphere of 95% oxygen-5% carbon dioxide. At the end of the incubation period the medium was removed by suction, the slices were washed with several changes of media, and the original solution and washings were pooled. The medium was then extracted with 2×10 ml. of chloroform, aliquots were pipetted into test tubes and evaporated to dryness in a water bath at 40° C.

Color determinations were performed on the dried aliquots as described. Details are supplied since they vary from the original methods in many cases.

Porter-Silber Reaction: Four ml. of a solution of 3 ml. of 60% sulfuric acid and 1 ml. of absolute methanol containing 65 mg.% phenylhydrazine were pipetted into each tube. The tubes were placed in a water bath at 60° C for 30 minutes for color development, and the color was then read in a spectrophotometer at 410 mμ. Blanks and standards were run in the usual fashion.

Blue Tetrazolium Reaction: To the dried extract was added 0.2 ml. of 95% ethanol followed by 0.15 ml. of ethanol containing 1.5 mg./ml. of BTZ. Tetraethylammonium hydroxide was added (0.15 ml. of a solution of 0.3 ml. of 10% TEA in 10 ml. of 95% ethanol) and the mixture was allowed to develop color in the dark for 20 min. The solution was then diluted to 5 ml. with 95% ethanol and the color intensity was read at 510 mμ in the spectrophotometer.

Gornall Reaction: One-half ml. of a solution of 3 parts methyl alcohol (absolute) and 1 part conc. HCl containing 1 mg./ml. of 2,4 dinitrophenylhydrazine was added to the dried aliquot. The tubes were then placed in a water bath at 60° C for 90 min. in the dark. After heating, the tubes were cooled and 0.5 ml. of 4N aqueous NaOH was added and rapidly mixed. Five ml. of absolute methanol were then added. Thirty minutes after addition of the methanol the color was read at 475 mμ in a spectrophotometer.

Ultra-violet Absorption: Five ml. of absolute methanol were added to the dried aliquot and the resulting solution was read in quartz cuvettes in the Beckman DU spectrophotometer at 240 mμ.

In all cases, precautions were taken to assure that the tissue and reagent blanks were low. The extraction process was checked in every run by extracting controls of medium and steroid with no slices and controls with steroid and slices previously inactivated by boiling. In addition, each steroid was incubated with several flasks of tissue from the same liver and with tissue from several livers. Cortisone was selected for comparison since in previous work (4) data had been obtained on the metabolism of this steroid in several hundred cases. This work established that (a) absorption on surfaces was minimal; (b) steroid lost from the media could not be recovered in slices indicating metabolism of the steroid; and, (c) the rate of metabolism was relatively constant over the incubation period. Each run consisted of several steroids and cortisone. The steroids were varied from day to day. Blanks were used for each steroid.

RESULTS

Methods: Four methods were selected to study alterations in structure which might occur in the steroid molecule during metabolism. The Porter-Silber (P-S) reaction was selected as it was specific for the dihydroxyacetone sidechain. Blue tetrazolium (BTZ) reaction was selected as it reacts

[3] The progesterone and 17-hydroxy progesterone were added in 0.4 ml. ethanol because of the low solubility of these compounds.

with an alpha ketone, but does not require the presence of a hydroxyl group at C_{17}. Absorption at 240 mμ (E-240) indicates the presence of a 3-keto-Δ^4 function in Ring-A. Decrease in optical density at 240 mμ measured reduction of positions 3 and/or 4. The Gornall reaction produces maximum color with the dihydroxyacetone sidechain as does the P-S, but in addition there is some contribution from the 11 position and Ring-A (5). The extinction coefficients for the steroids investigated in this problem are presented in Table 1.

Metabolism of the Steroid Molecule: The data obtained on the metabolism

TABLE 1. THE MOLAR EXTINCTION COEFFICIENTS OF VARIOUS STEROIDS AS DETERMINED AT THE OPTIMUM WAVELENGTH FOR EACH REACTION IN A BECKMAN DU SPECTROPHOTOMETER (1 CM. LIGHT PATH)

Steroid	Porter-Silber, 410 millimicrons	Blue tetrazolium, 510 millimicrons	Max $E_{240\ m\mu}$	Gornall, 475 millimicrons
Hydrocortisone	18,400	19,600	19,900	39,600
Δ^1 Hydrocortisone (Prednisolone)	19,700	21,500	19,300	40,800
Δ^1,9α-Fluoro-hydrocortisone	19,800	20,900	22,200	39,500
9α-Fluoro-hydrocortisone	21,300	22,400	22,300	37,500
2-Methyl Hydrocortisone	18,400	21,700	18,700	29,400
2-Methyl-9αFluoro-hydrocortisone	18,900	22,100	18,300	29,000
Cortisone	23,400	22,700	18,000	36,500
Δ^1 Cortisone (Prednisone)	20,300	21,200	19,300	43,200
11-Desoxycortisone (S)	19,500	18,700	23,200	36,000
Desoxycorticosterone	—	20,000	20,400	22,800
Corticosterone	—	21,800	23,500	33,500
Progesterone	—	—	22,000	18,500
17-Hydroxyprogesterone	—	—	20,700	22,700
Tetrahydrocortisone	21,800	21,800	—	21,900

of steroids in liver slices are presented in Table 2. The P-S reaction and the Gornall reaction varied in the same direction. The Gornall reaction indicated consistently greater metabolism which may be explained by the fact that reduction of ketone in Ring-A was proceeding simultaneously with loss of function in the sidechain. In almost every case, loss of structure as measured by P-S reaction was similar to that measured by BTZ reduction. As the loss of a 17-hydroxyl group would have been reflected in the P-S reaction but not in the BTZ reaction, we must conclude that saturation probably occurred at the 20-ketone position. This has also been reported by deCourcy (12), and is further demonstrated by the fact that DOC and corticosterone lost reducing activity in the sidechain when no 17-hydroxyl group was present. The Ring-A reduction was in most cases larger than the extent of changes in the sidechain. This confirms previous finding from this laboratory (4), and those of Dorfman and Ungar (8) who state that the reduction of the Ring-A must precede the changes in the sidechain.

Metabolism of Specific Steroids: The data in Table 2 also demonstrate that the rate of destruction of the steroid sidechain in the liver was but little affected by the addition of a structure normally foreign to the molecule.

TABLE 2. THE METABOLISM OF VARIOUS SUBSTITUTED STEROIDS IN LIVER SLICES. IN ALL CASES, 250 MG. OF LIVER TISSUE WERE INCUBATED WITH 500 μG. OF STEROID IN 3 ML OF KREB'S BICARBONATE BUFFER FOR ONE HOUR. THE RESULTS ARE EXPRESSED AS STEROID LOST FROM THE MEDIUM UNDER THESE CONDITIONS. TISSUE BLANKS (15–20 MG./250 MG. TISSUE/HR) WERE PREVIOUSLY SUBTRACTED

Steriod	No. of tests	Micrograms of steroid Lost/250 mgs. tissue/hr.			
		Porter-Silber reaction	Blue tetrazolium reaction	Max $E_{240\ m\mu}$	Gornall reaction
Hydrocortisone	52	98 ± 10*	104 ± 4	145 ± 6	88 ± 12
Δ^1-Hydrocortisone (Prednisolone)	21	101 ± 8	105 ± 7	92 ± 6	106 ± 5
Δ^1-9α-Fluoro-hydrocortisone	18	95 ± 5	95 ± 7	117 ± 9	104 ± 5
9α-Fluoro-hydrocortisone	15	92 ± 6	100 ± 4	115 ± 6	131 ± 10
2-Methyl hydrocortisone	24	90 ± 6	105 ± 9	100 ± 8	79 ± 10
2-Methyl,9α-Fluoro-hydrocortisone	27	100 ± 9	107 ± 6	102 ± 6	100 ± 8
Cortisone	12	126 ± 8	136 ± 12	147 ± 14	146 ± 18
Δ^1-Cortisone (Prednisone)	21	83 ± 9	95 ± 9	92 ± 6	81 ± 7
11-Desocy Cortisone (S)	18	175 ± 14	165 ± 10	161 ± 18	—[2]
Desoxycorticosterone	12	—[1]	232 ± 34	304 ± 40	310 ± 25
Corticosterone	12	—[1]	175 ± 24	268 ± 51	—
Progesterone	15	—[1]	—[1]	132 ± 4	82 ± 2
17-Hydroxy Progesterone	15	—[1]	—[1]	116 ± 23	162 ± 8
Tetrahydrocortisone	12	147 ± 21	149 ± 18	—[1]	—[2]

* Standard Deviation.
[1] Does not react.
[2] Not done.

This is a point which is in controversy. Slaunwhite (9) has pointed out that prednisolone was metabolized almost as readily as F in the human and we confirm these results. Liddle (10), on the other hand, has stated that 2-methyl hydrocortisone was long acting because it cannot be inactivated by liver extracts, and Tompkins *et al.* (11) found that prednisolone cannot be metabolised by tissue extracts. In our experiments there appeared to be a depressed but not suppressed metabolism of Ring-A when substituents were added to the ring. Tompkins (14) has recently investigated the rate of reduction of the Ring-A in eight steroids similar to those reported here. An acetone dried enzyme preparation of liver was employed and all steroids were reduced except 2-methylhydrocortisone. Progesterone was reduced in smaller amounts than the adrenal cortical steroids. Except for these two steroids, our results agree with those of Tompkins.

For reasons which are not clear, the other naturally occurring steroids are inactivated much more rapidly by the liver than is hydrocortisone or cortisone. This may be the reason for the potency of the latter compounds. The high rate of catabolism of tetrahydrocortisone has been reported by Glenn (12) in liver subcellular fraction. The higher biological potency of the substituted steroids is unexplained. Slaunwhite (9) has claimed that the increased activity is not due to retention in the plasma, slow absorption in the gut, or increased absorption by the tissues. From our experiments, it does not appear to be due to decreased destruction by the liver, although reduction of the Ring-A is depressed.

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COMPARATIVE POTENCY OF GROWTH HORMONE PREPARATIONS IN ALKALI OR ACID

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ABSTRACT

Growth hormone preparations obtained from beef pituitary through alkali extraction (methods of Li and Wilhelmi) were compared for growth activity in hypophysectomized rats with acid extracted porcine growth hormone (method of Raben). The alkali extracted preparations showed reduced activity when assayed in acid media. The reduced potency was shown to result from storage at the acid pH, rather than from inactivity at the time of injection. Hydrochloric acid was shown to be more damaging than acetic acid of comparable pH. Somatotropic preparations obtained from beef or pork pituitary through acid extraction showed no difference when assayed in basic or acidic media. It was concluded that the differential effect of pH on Li or Wilhelmi growth hormone as compared to Raben type preparations is not due to the gland source, but rather to the method of preparation.

MOST methods for obtaining growth hormone make use of extraction of beef anterior pituitary tissue with alkali. This is the case with the classical procedures of Li, Evans and Simpson (1), Wilhelmi, Fishman and Russell (2) and more recent methods (3). The studies of Li and Papkoff (4) have shown that purified beef growth hormone quickly loses some of its biological activity if placed in glacial acetic acid or even 0.1N acetic acid. Furthermore, Ellis *et al.* (5) have reported that loss of biological activity and electrophoretic heterogeneity is most pronounced if the growth hormone is treated with mineral acids, like hydrochloric acid.

In contrast to this is the work of Evans *et al.* (6) and more recently Raben and Westermeyer (7) who obtained growth hormone preparations with marked biological activity from acetic acid extracts of pituitaries. Moreover, Raben's preparation showed no loss of activity after storage in 0.1N acetic acid for two days at room temperature and up to six weeks at refrigeration temperature.

In the present investigation the potency of various growth hormone preparations was compared when dissolved in alkali and acid, and to rule out species differences, the activities of beef and pork material obtained by identical procedures were also compared.

MATERIALS AND METHODS

Three alkali extracted beef growth hormone preparations were used. One was obtained from Dr. Li and another from Dr. Wilhelmi. The third was the United States

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Pharmacopeia (U.S.P.) Growth Hormone Reference Standard. The acid extracted growth hormone preparations were obtained at The Wilson Laboratories from pork or beef glands, according to the procedure of Raben and Westermeyer (7).

Assays for somatotropic activity were carried out by the 10-day growth test in hypophysectomized, male, 100 gm. rats. The animals were received from Hormone Assay Laboratories and were fed a diet containing 25% casein.[1] Plateaued rats failing to gain or lose over 5 gm. during the third post-operative week were selected for assay. The rats were distributed at random into groups of 5 to 7. Injections were made subcutaneously, 0.5 cc. per rat per day, for 10 days. Final body weights were determined on the day after the last injection.

In a first experiment, each growth hormone preparation was assayed against itself when injected in alkaline and acid solution. This was made by dissolving 10 mg. in 0.05N HCl or 0.05N NaOH and immediately diluting with water to injected concentrations. These solutions were refrigerated during the 10-day test period. In a second experiment, the effect of an organic acid, a mineral acid and alkali was compared. This was made by dissolving 50 mg. of each growth hormone in 5 cc. samples of 0.03N HCl (pH 2.7), 2.0N acetic acid (pH 2.8) and 0.07N NaOH (pH 11.0). The solutions again were refrigerated during the test period. For assay, appropriate dilutions were made with distilled water and the pH adjusted to 4.0 before injection.

TABLE 1. COMPARISON OF VARIOUS GROWTH HORMONE PREPARATIONS ASSAYED IN ACID AND ALKALI

Growth hormone preparation and source	Weight gain (gm.) – Mean ± S.E.*		Significance of difference
	pH 8.0	pH 4.0	
Wilhelmi *et al.* bovine	21.0 ± 1.6	12.0 ± 1.8	P .01
Li *et al.* bovine	18.8 ± 1.2	13.8 ± 1.5	P .05
U.S.P. Standard bovine	17.6 ± 1.1	10.8 ± 1.1	P .05
Raben *et al.* porcine	13.6 ± 0.8	14.4 ± 1.6	not sig.
Raben *et al.* bovine	10.0 ± 1.2	7.0 ± 1.2	not sig.

* Dose: 25 μg. per day for 10 days—5 rats per group.

RESULTS

The results of comparing each type of growth hormone against itself in alkaline or acid solution are presented in Table 1. The data show that the three growth hormone preparations obtained from beef pituitary by extraction with alkali (methods of Wilhelmi *et al.*, Li *et al.* and U.S.P. Growth Hormone Standard) show decreased activity when dissolved and administered in acid. The beef and pork preparations obtained by acid extraction (method of Raben *et al.*), however, show the same activity when dissolved in alkali or acid.

The effect of storing at various pH conditions is shown in Tables 2 and 3. Since in these experiments all solutions were injected at the same pH, the differences in activity may be attributed solely to the method of storing,

[1] The composition of the diet in percent was: casein 25, milk powder 10, yeast 10, desiccated liver 3, salts IV (8) 2, sucrose 45 and corn oil 5.

TABLE 2. EFFECT OF STORING AT VARIOUS pH CONDITIONS ON BEEF GROWTH HORMONE OBTAINED BY ALKALINE EXTRACTION (METHOD OF WILHELMI *et al.*)

HCL Stored at pH 2.7 Injected at pH 4.0	Acetic acid Stored at pH 2.8 Injected at pH 4.0	NaOH Stored at pH 11.0 Injected at pH 4.0
Weight gain (gm.) – Mean ± S.E.*†		
10.0 ± 1.8	15.4 ± 1.5	22.5 ± 2.1

* Dose: 25 μg. per day for 10 days.
† Means significantly different by analysis of variance (F-test at P .01).

exclusive of effects at the site of injection. Alkali extracted beef growth hormone (method of Wilhelmi *et al.*) shows decreased activity when stored in acid, and the decrease in potency is more pronounced in hydrochloric acid then in acetic acid of equivalent pH (Table 2). Acid extracted preparations (method of Raben *et al.*), whether from bovine or porcine source, show no difference in activity after storage in hydrochloric acid, acetic acid or sodium hydroxide (Table 3).

DISCUSSION

The present data indicate the danger of conducting assays of highly purified growth hormone in acid solution, since preparations such as obtained by the methods of Li or Wilhelmi, show definite loss of activity when stored in acid. The present findings support previous reports (4, 5) showing decreased stability of alkali extracted beef growth hormone exposed to acid. Our finding that mineral acids such as hydrochloric exert a more pronounced effect on Wilhelmi type growth hormone than organic acids such as acetic, confirms the work of Ellis *et al.* (5) who, using their own preparation, reported a loss in activity of 54% in hydrochloric acid and only 15–20% in acetic acid of comparable pH under the same conditions.

The decreased activity of the U.S.P. Growth Hormone Reference Standard in acid solution has been previously reported (9). According to its distributors, the U.S.P. material was prepared from beef glands by modification of the Wilhelmi method.

The Raben type preparations, on the other hand, show the same activity

TABLE 3. EFFECT OF STORING AT VARIOUS pH CONDITIONS ON GROWTH HORMONE OBTAINED BY ACID EXTRACTION (METHOD OF RABEN *et al.*)

Source of hormone	HCl Stored at pH 2.7 Injected at pH 4.0	Acetic acid Stored at pH 2.8 Injected at pH 4.0	NaOH Stored at pH 11.0 Injected at pH 4.0
	Weight Gain (gm.) – Mean ± S.E.*†		
pork	13.8 ± 0.7	14.0 ± 1.6	16.7 ± 1.1
beef	13.4 ± 1.2	14.4 ± 0.8	15.5 ± 1.9

* Dose: 50 μg. per day for 10 days.
† Means not significantly different by analysis of variance (F-test at P .05).

when dissolved in alkali or acid. This may be explained by the fact that the product, being acid extracted, is no longer adversely affected by acid treatment as used in these experiments.

Differences between the acid-lability of Raben type pork somatotropin and Wilhelmi or Li type beef somatotropin cannot be explained in terms of animal source. Growth hormone prepared by the Raben procedure from beef pituitaries has equivalent activity in acid or alkali, in the same manner as pork pituitary derived Raben growth hormone.

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THE EFFECTS OF VARIOUS ESTROGENS AND STEROID SUBSTANCES ON SEX DIFFERENTIATION IN THE FOWL

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ABSTRACT

An account is given of the effects of the immersion of 4108 fertile, preincubation, hen's eggs into ethanol solutions of diethylstilbestrol (DES), and of 1524 such eggs into isopropyl alcohol solutions of DES. The percentage of hatched genetic male chicks showing feminization at hatching increased, on the average, with increasing concentrations of DES in the dipping solutions and, up to a point, with increased immersion time. Occasional small percentages of genetic females were virilized by the ethanol solutions, but were not seen in the experiments were isopropyl alcohol was the solvent. Maximal morphological reversions were observed in isopropyl alcohol solutions of DES. Ethinyl estradiol in ethanol solution also induced significant percentages of feminization, while testosterone in ethanol irregularly induced limited masculinization. Allopregnan-21-ol-3,20-dione acetate either in ethanol or in isopropyl alcohol reduced the percentage of eggs hatching and tended to be differentially lethal to female embryos. Of two non-steroidal estrogens, the methyl ether of allenolic acid and 2-methyl-3-ethyl-4-(p-anisyl)-8-Δ^3-cyclohexene-1-carboxylic acid, the latter at relatively low dose was partially effective in inducing feminization. Two 19-nor steroids had no androgenic or estrogenic effects; a third, 17-ethinyl-estra-3,5-diene-3,7-diol diacetate at high dose was lethal to all embryos. 17-Methyl testosterone, progesterone, and Δ^5-pregnenolone induced a limited percentage of masculinization, the latter two tending to reduce hatching percentages at higher doses. Pregnane-3α,20α-diol, at the dosage used, reduced hatching percentage but did not affect morphological sex differentiation. The corticosteroids, with the exception of 1-dehydrocortisol at low dose, were lethal to all embryos in the doses tested, and 1-dehydrocortisol had no effect on sex differentiation.

A number of genetic males hatched with female genitalia as a result of estrogen treatment of the egg reverted to male appearance and behavior, with well developed testes, in twelve to sixteen weeks. The removal of the right testis of feminized male chicks did not inhibit regression to the male type. Maintaining feminized, right gonadectomized males on stilbestrol-enriched food for many weeks maintained female appearance and behavior, but the left gonad remained sterile.

A RAPID method recently described for the introduction of various substances into the intact, fertile, chicken egg (1) led us to investigate the utility of this method for determining the effects of a number of estrogens and steroids on the course of sex differentiation in the chick embryo. Previous studies have involved the injection of steroids into the egg, usu-

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[1] Aided by a grant from the Vineland Poultry Laboratories, Vineland, New Jersey.

ally necessitating the removal of a portion of the shell and operation under sterile conditions (2, 3). The Seltzer method obviates the need for surgical conditions, and the shell is maintained intact.

MATERIALS AND METHODS

Fertile eggs were obtained from a breeder of the sex-linked head spot strain of chickens. Following treatment, the eggs were incubated in a conventional chicken egg incubator at 39° C until hatching. At hatching, the sex of each live chicken hatched was determined by a commercial sexor who had no knowledge of the type of treatment employed, and who depended on the appearance of the external genitalia for "sexing." Since genetic sex was marked by the presence or absence of the head spot, departure from the genetic sex could be determined at hatching. A number of "sex-reversed" birds from treated eggs and controls were maintained for periods up to several months to determine the degree of subsequent development toward maleness or femaleness.

The treatment designed to permit penetration of the steroids into the fertile egg involved dipping the pointed end of the egg to approximately one-half its length (unless otherwise noted) in a solution of the steroid kept at 12° C. The eggs, previous to dipping, were at room temperature (23°–25° C.) With the use of a properly designed egg-holding tray, it was possible to immerse ten eggs simultaneously into the steroid solutions. The nature of the solvent solution and the periods of immersion of eggs are given in the accompanying tables. Immediately after immersion the eggs were kept at room temperature and transferred to a hatching incubator at 18 hours after treatment.

RESULTS

Since estrogen administration is known to cause morphological sex-reversal to femaleness (1, 2, 3), we concentrated our initial studies on determining the optimum conditions for the estrogen effect by the Seltzer immersion method. Using 95% ethanol as the solvent to which was added (after Seltzer) 0.2 mg. riboflavin and 0.1 mg. $FeSO_4$ per egg dipped, we examined the effects of various concentrations of diethylstilbestrol and of various periods of immersion upon the sex at hatching. The data of these experiments are presented in Table 1.[2] These data demonstrate: (a) an average increase in the proportion of feminized males to a maximum at the highest dosage of stilbestrol used, (b) a tendency for a lower proportion of such morphological reversals at the 5 second dipping time at the lowest stilbestrol concentration, (c) a tendency for somewhat lower hatching percentages at the 20 mg.% and the 40 mg.% stilbestrol concentrations, and (d) an occasional occurrence of genetic females with male processes.

In Table 2 we present data on the rate of change in external genitalia using isopropyl alcohol as the steroid solvent. Here we observe: (a) a higher average percentage of feminized males than with ethanol as the solvent, (b) a higher average hatching percentage than in the ethanol experiments

[2] In this and subsequent tables, the column headed "% ♂ with ♀ processes" gives the reversed males as a percent of the total males, the column headed "% ♀ with ♂ processes" the reversed females as a percent of the total females, and "% ♀" the female percentage of the total hatch.

TABLE 1. THE EFFECTS OF THE IMMERSION OF FERTILE CHICKEN EGGS INTO 95% ETHANOL SOLUTIONS OF DIETHYLSTILBESTROL (DES). 0.2 MG. RIBOFLAVIN AND 0.1 MG $FeSO_4$ PER EGG DIPPED ADDED TO THE DIPPING SOLUTION

DES conc., mg./100 ml.	Immersion time, seconds	No. of eggs set	% hatching	% ♂ with ♀ processes	% ♀ with ♂ processes	% ♀
10	5	180	70.5±3.4*	11.1± 1.2	10.9±4.2	43.2±4.4
10	5	150	81.3±3.2	29.4± 5.5	—	44.3±4.5
10	6	180	68.3±3.5	44.8± 5.9	—	45.6±4.5
10	10	120	71.6±4.1	38.6± 7.3	4.8±3.3	48.8±5.4
10	10	150	75.4±3.5	32.8± 5.9	—	43.3±4.7
10	10	180	73.9±3.3	81.1± 4.5	—	56.6±4.3
10	10	180	71.1±3.4	10.4± 3.5	—	40.3±4.3
10	10	180	72.2±3.3	82.0± 4.7	—	48.4±4.4
10	10	175	77.1±3.2	81.8± 4.4	—	42.9±4.3
10	10	180	77.8±3.1	82.4± 4.4	—	47.3±4.2
10	10	178	83.7±2.8	56.5± 6.0	—	53.7±4.1
10	10	180	81.1±2.9	46.8± 5.7	—	47.3±4.1
10	20	178	73.6±3.3	71.4± 5.7	—	51.8±5.8
20	6	180	57.2±3.7	28.3± 6.6	12.3±4.4	55.3±4.9
20	10	180	72.7±3.3	51.7± 6.6	—	55.7±4.3
20	10	96	65.6±4.9	63.6± 8.4	—	47.6±6.3
20	10	84	64.4±5.2	44.0± 9.9	—	53.7±6.8
20	10	90	71.0±4.8	42.4± 8.6	9.7±5.3	48.4±6.3
20	10	178	77.0±3.2	82.6± 4.6	—	49.6±4.3
20	10	90	74.5±4.6	89.1± 5.1	—	44.8±6.1
20	10	180	79.5±2.8	65.0± 6.2	—	58.1±4.1
20	10	180	77.3±3.1	77.0± 5.0	—	49.7±4.2
20	20	90	62.3±5.1	33.3±10.3	14.3±5.9	62.5±6.5
20	20	90	78.9±4.3	83.3± 6.2	—	49.3±5.9
40	10	179	76.2±3.2	93.1± 3.3	—	57.4±4.2
40	10	180	71.7±3.4	98.4± 5.2	—	52.6±4.4

* The standard errors of the percentages in this and succeeding tables are derived from the formula $\sqrt{(p \times q)/n}$, where p = the observed percentage, $q = 100 - p$, and n is the number of observations (e.g., the number of eggs set under "% hatching," the number of animals under "% ♂ with ♀ processes," etc.).

at the higher stilbestrol concentrations, (c) no particular difference in either change in external genitalia or hatching percentages when complete immersion of the eggs was practiced, (d) no differences in male feminization rate at the 40 mg. per 100 ml. concentration at dipping times of 5 seconds and 10 seconds, and (e) no incidence of female masculinization.

In Table 3 we present data on the effects of three steroids and a combination of two of them upon hatching percentages and the degree of morphological sex reversal. It will be seen that: (a) ethinyl estradiol is effective in such "reversal" at a concentration of 5 mg. per 100 ml., and that the average per cent of feminization is somewhat less than with 10 mg. of diethylstilbestrol in ethanol (compare Table 1), (b) in only one instance did testosterone, and that at a lower dose, appear to cause some virilization, (c) allopregnanedione acetate, at the dosage used, severely reduced the hatching percentage with no effect on the external genitalia, and (d) the combination of allopregnanedione with testosterone seemed further to reduce the hatching percentage with a 20% incidence of virilization and a significant lowering of the percentage of females hatched.

TABLE 2. THE EFFECTS OF THE IMMERSION OF FERTILE CHICKEN EGGS INTO ISOPROPYL ALCOHOL SOLUTIONS OF DIETHYLSTILBESTROL (DES)

DES conc., mg./100 ml.	Immersion time, seconds	No. of eggs set	% hatching	% ♂ with ♀ processes	% ♀ with ♂ processes	% ♀
10	10	90	71.2±4.8	91.9±4.5	—	42.3±6.2
10	10	90	73.3±4.7	90.0±5.5	—	54.6±6.1
40	5	88	75.5±4.6	100.0±0.0	—	44.3±6.1
40	5[1]	88	81.8±4.1	97.0±2.9	—	52.7±5.9
40	5	180	72.3±3.3	98.4±1.6	—	53.8±4.4
40	5	180	67.8±3.5	98.6±1.4	—	41.8±4.5
40	5[1]	90	77.8±4.4	100.0±0.0	—	47.2±6.0
40	5[1]	89	75.3±4.6	100.0±0.0	—	59.7±6.0
40	5[1]	180	75.5±3.2	91.0±3.5	—	51.5±4.3
40	10	179	74.9±3.3	98.5±1.5	—	51.5±4.3
40	10	180	76.8±3.1	94.7±2.6	—	45.6±4.2
40	10	90	72.2±4.7	100.0±0.0	—	52.3±6.2

[1] Total immersion of eggs.

In Table 4 we list two estrogens and a variety of steroids dissolved in isopropyl alcohol, with the hatching percentages and percentages of "sex reversal" obtained. The notable features of these data are: (a) the ineffectiveness of I as a feminizing agent and its inhibition of hatching, (b) the effectiveness of II at a relatively low dose along with a normal hatching percentage, (c) the ineffectiveness of the 19-nor steroids III, IV, and V, and the lethal effect on the embryos of V, (d) the lack of activity of VI, (e) the activity of VII which at the lower dosage used, partially induces a virilization of females, and at the higher dose appears to act partially in both directions, (f) the activity of VIII, IX, and X as virilizers along with a tendency, particularly at the higher doses of VIII and IX, to reduce hatching percentages, (g) the failure of VII plus VIII at the dose combination used to increase the percentage virilized, (h) a tendency of VII, VIII,

TABLE 3. THE RESULTS OF THE DIPPING OF FERTILE CHICKEN EGGS INTO 95% ETHANOL SOLUTIONS OF VARIOUS STEROIDS. RIBOFLAVIN 0.2 MG. AND $FeSO_4$ 0.1 MG. PER EGG ADDED TO THE DIPPING SOLUTION. IMMERSION TIME 10 SECONDS

Compound	Concentration, mg./100 ml.	No. of eggs set	% hatching	% ♂ with ♀ processes	% ♀ with ♂ processes	% ♀
Ethinyl estradiol	5	90[1]	71.1±4.8	59.2±9.5	—	57.8±6.2
Ethinyl estradiol	5	90	70.0±4.8	35.5±9.7	—	50.7±6.3
Ethinyl estradiol	5	85	71.7±4.9	20.7±7.5	—	52.4±6.4
Testosterone	10	90	73.3±4.6	2.8±2.8	26.7± 8.1	45.4±6.1
Testosterone	10	179	81.0±2.9	—	—	54.5±4.1
Testosterone	100	90	68.8±4.9	—	—	48.4±6.4
Testosterone	100	90	77.8±4.4	—	—	35.7±5.7
Allopregnane-21-ol-3, 20-dione acetate	135	176	34.6±3.6	—	—	42.6±6.3
Testosterone + Allopregnane-21-ol-3, 20-dione acetate	100 + 94	180	20.0±3.0	—	20.0±12.7	27.8±4.1

[1] 5 seconds immersion time.

TABLE 4. THE EFFECTS OF VARIOUS STEROIDS ON HATCHING PERCENTAGE AND PERCENTAGE O MORPHOLOGICAL SEX REVERSAL. ALL STEROIDS DISSOLVED IN ISOPROPYL ALCOHOL. IMMERSION TIME 10 SECONDS

Steroid No.	Name	Conc., mg./100 ml.	No. of eggs set	% hatching	% ♂ with ♀ processes	% ♀ with ♂ processes	% ♀
	Control	—	90	77.8±4.4	—	—	48.6±5.9
I	Methyl ether of allenolic acid	50	180	32.2±3.5	—	3.2± 3.2	53.5±6.6
II	2-methyl-3-ethyl-4-(p-anisyl)8-Δ^3-cyclohexene-1-carboxylic acid	8	87	74.7±4.7	62.5±8.6	—	50.8±6.2
III	17-ethinylestraeneolone	40	90	69.9±4.8	—	—	44.4±6.3
IV	17-ethinyl-19-nortestosterone	40	90	76.6±4.5	—	—	49.3±6.0
V	17-ethinyl-estra-3,5-diene-3,17-diol diacetate	100	90	—	—	—	—
VI	17-ethinyl androstenediol	100	180	76.8±3.2	—	—	45.6±4.2
VII	17-methyl testosterone	20[1,2]	89	83.1±4.0	—	20.9± 5.9	64.9±6.0
	17-methyl testosterone	20	180	63.3±3.6	—	7.1± 4.0	36.8±4.5
	17-methyl testosterone	20	90	68.9±4.9	—	22.7± 8.9	35.5±6.1
	17-methyl testosterone	100	180	78.9±3.0	17.5±4.3	12.9± 4.3	43.9±4.2
	17-methyl testosterone plus progesterone (VIII)	8 / 40	180	49.5±3.7	—	27.8± 7.5	40.4±5.2
VIII	Progesterone	20[1]	90	53.4±5.3	—	29.2± 9.3	50.0±7.2
	Progesterone	20	90	86.6±3.6	2.6±2.6	2.6± 2.6	50.0±5.7
	Progesterone	80	179	29.1±3.4	—	33.3±15.7	17.3±5.3
IX	Δ^5-pregnenolone	50	90	81.1±4.1	—	19.4± 7.1	42.4±5.8
	Δ^5-pregnenolone	100	180	51.1±3.7	—	20.5± 6.5	42.4±5.2
X	Δ^5-pregnenolone glucoside	50	90	77.8±4.4	—	27.3± 7.8	47.2±6.0
	Δ^5-pregnenolone glucoside	50	180	68.9±3.5	—	—	49.2±4.5
XI	Pregnane-3α,20α-diol	50	180	31.7±3.5	—	—	47.4±6.6
XII	Allopregnane-21-ol-3,20-dione	50	90	53.3±5.3	—	—	39.6±7.1
XIII	Corticosterone	5	180	1.1±0.8	—	—	—
	Corticosterone	10	90	—	—	—	—
	Corticosterone	25	90	—	—	—	—
	Corticosterone	50	180	—	—	—	—
XIV	Cortisol	50	180	—	—	—	—
XV	1-dehydrocortisone	4	89	—	—	—	—
XVI	1-dehydrocortisol	4	90	53.3±5.3	—	—	52.2±7.2

[1] 5 seconds dipping time.
[2] Complete immersion.

XII, and perhaps IX to reduce the percentage of genetic females hatched: e.g., note percentages of females of 36.8, 35.5, 17.3, and 39.6, which in some instances are significantly below the control value of 48.6, (i) the inactivity of XI and XII at the dosage used, with a reduced hatching percentage, and (j) the lethal effect of the corticosteroids (XIII, XIV, XV, and XVI) tested, with a somewhat reduced lethality of XVI, unaccompanied by any reversal effect.

A number of the "reversed" genetic male chicks were raised for varying periods of time as indicated in Table 5. If these birds were fed on regular

TABLE 5. OBSERVATIONS OF THE DEVELOPMENT OF "SEX-REVERSED" GENETIC MALE CHICKS

Expt. No.	No. of birds	Treatment of eggs	Operation	Diet	Time observed, weeks	Remarks
1	16	40 mg DES/100 ml. isopropyl alcohol, 5 sec. dip	none	Chick mash	16	Some reversion at 12 weeks; all reverted at 16 weeks; active testes
2	15	20 mg. DES/100 ml. isopropyl alcohol, 10 sec dip	right gonadectomy at 12 days of age	Chick mash	16	All reverted at 16 weeks; sterile gonads
2	15	20 mg. DES/100 ml. isopropyl alcohol, 10 sec. dip	right gonadectomy at 12 days of age	Chich mash +250 mg. DES per kilogram	28	Maintained female combs and behavior, gonads sterile; injection of sheep pituitary FSH +chorionic gonadotrophin to the 33rd week in 2 birds led to no gonad stimulation or gametogenesis
4	10	8 mg. compound II per 100 ml.	right gonadectomy at 7 days of age	Chick mash	11	All regressed at 11 weeks; limited spermatogenesis in several gonads examined at 11 weeks

chick mash, return to normal male appearance with active testes occurred by 16 weeks of age (Experiment 1). Birds from which the right testis was removed and which were fed on the regular diet all reverted to male appearance (Experiments 2 and 4) in 11 to 16 weeks, and either had sterile left gonads (Experiment 2) or a limited degree of spermatogenesis in the left gonad (Experiment 4). Right gonadectomized birds fed on the regular diet plus DES maintained the appearance and behavior of females but the left gonad was sterile and it could not be stimulated by mammalian gonadotropin administration.

DISCUSSION

Treatment of eggs by the Seltzer method clearly results in the penetration of certain estrogens and of various steroids as judged by the degree of morphological sex reversal induced in both genetic sexes, the reduction in hatching percentages in certain instances, and the outright lethal effects in other instances. Under the conditions of the experiments described, the alteration in external genitalia evident at hatching is not reflective of a gonadal sex reversal since the sixteen "reversed" genetic males raised to 16 weeks of age had normally active testes. Nor does the left testis appear to have been altered in the female direction since it either remains as a sterile organ when the contralateral testis is removed at an early age or even develops a limited amount of spermatogenetic activity. The effects of the estrogens therefore would appear to be exerted directly on the Mullerian duct rudiments in the embryos. The fact that these effects are obvious at hatching suggests a fairly long term maintenance of activity since the absorption of the estrogens occurred in fertilized eggs in which these rudiments do not develop for a number of days after treatment; furthermore, they are maintained in apparently normal state until hatching.

Masculinization of the female has not been accomplished as easily or to the same extent as reversal to female type. Although a variety of steroids are partially effective for this purpose, i.e., testosterone, 17-methyl testosterone, progesterone, Δ^5-pregnenolone and its glucoside, no more than one-third of the genetic females have exhibited male genitalia (Table 4). Furthermore, at higher dosages these compounds tend to be lethal to the embryo, with an irregular tendency to differential mortality of the female embryos. Judging by the condition of the embryos in the unhatched eggs, these lethal effects occur at various times during development, whereas the lethal effects of the corticosteroids are generally exerted on the germ practically immediately. Unfortunately, due to a laboratory accident, we have thus far not raised any virilized genetic females for more than a few days beyond hatching, so we cannot describe their development.

There is no need to review here the voluminous literature on the effects of steroids on sex development in the fowl (cf. 2, 3). It suffices here to point out that our data are in accord with those generally reported for com-

pounds injected directly into the egg, although to our knowledge the androgenic effects here reported of progesterone and Δ^5-pregnenolone on the chick embryo have not been reported elsewhere. It is evident, we believe, that the immersion method does offer opportunity for easy and rapid medication of the chick embryo. We hope, in future publications, to demonstrate further interesting applications.

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ALTERATIONS IN SODIUM AND POTASSIUM METABOLISM FOLLOWING HIND LEG FRACTURE IN THE RAT: ROLE OF THE ADRENAL CORTEX[1]

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ABSTRACT

Adrenalectomized rats, maintained with saline and allowed food *ad libitum*, showed a positive sodium balance and a negative potassium balance following fracture of the bones of both hind legs. Qualitatively similar results were obtained in similarly treated sham adrenalectomized rats. These findings were confirmed and amplified using force-fed rats. It has been demonstrated that in force-fed adrenalectomized rats on a high salt intake there was a loss of potassium from uninjured tissues as well as from tissues which were mechanically damaged by the fracture procedure.

SEVERE tissue damage is frequently followed in man and experimental animals by retention of sodium, chloride and water (1–6) and by negative potassium and nitrogen balances (2–9). In addition, evidence has been presented for an increased activity of the adrenal cortex following trauma (1, 4, 5, 10–15). These observations coupled with the well known actions of the adrenocortical steroids on the kidney, i.e., sodium retention and increased excretion of potassium and nitrogen, have led certain investigators to suggest that the metabolic aberrations of trauma are a direct result of an increased secretory activity of the adrenal cortex. However, this view has been challenged.

Ingle *et al.* (2) have shown that force-fed adrenalectomized rats maintained with constant daily injections of adrenocortical extract exhibited urinary retention of sodium and chloride and increased urinary excretion of potassium and nitrogen when subjected to fracture of the bones of both hind legs. These metabolic changes were qualitatively and quantitatively similar to those seen in force-fed sham adrenalectomized rats subjected to fracture. Ingle *et al.* (2) concluded that the adrenal cortex does not play a causal but rather a supportive role in the development of the metabolic aberrations of trauma. However, even a supportive role for the adrenal cortex has been placed in doubt by the results of Noble and Toby (9) who found evidence for increased protein catabolism in traumatized, adrenalectomized rats maintained with saline.

The purpose of the work which is to be presented is to define more clearly

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the role of the adrenal cortex in the changes in electrolyte metabolism which follow bone fracture in the rat.

METHODS

Male albino rats weighing from 200 to 300 grams were adrenalectomized or sham adrenalectomized under ether anesthesia. Ten days later the rats were placed in individual metabolism cages for the collection of urine. In the first series of experiments the rats were fed *ad libitum* (Friskies dog food cubes) and given 0.9% sodium chloride solution to drink. The intake of food and saline was measured. In all subsequent experiments the rats were force-fed. Ingle's moderate carbohydrate diet (2) was used with the following modifications: Sodium caseinate was substituted for casein; the diet was supplemented with 50 gm. of sodium chloride; 1835 ml. of water were added to the mixed dry ingredients and oil. The animals were given distilled water to drink. After a suitable control period the femurs, tibias and fibulas of both hind legs were broken under ether anesthesia. A skin incision was made over the femur which was broken with a wire cutter. The incision was then closed with wound clips. The tibias and fibulas were broken manually. Blood samples were obtained from the abdominal aorta of the anesthetized rat.

Tissue samples were dried in a vacuum oven overnight at 105 to 110° C for the determination of water content. Fat was then extracted from the tissues by the method of Hastings and Eichelberger (16). The dried defatted muscle was ground to a powder and extracted for at least 48 hours at room temperature with 0.75N nitric acid. The dried bone powder (the bones were not defatted) from both hind legs of one rat was dissolved by placing in 10 ml. of concentrated nitric acid overnight at room temperature. All tissue extracts and digests were filtered[2] and appropriately diluted for analysis of sodium and potassium. These analyses were performed by internal standard flame photometry. The standard solutions used for the bone sodium analyses contained calcium in approximately the same concentration as the diluted bone digests. Calcium containing standards were found to be unnecessary for the bone potassium analyses. At the end of each experiment the adrenalectomized rats were either autopsied for the presence of adrenal regrowths or placed on standard stock diet without supplemental sodium chloride. The data from animals which had demonstrable regrowth of adrenal tissue or survived two weeks without supplemental salt were discarded.

RESULTS

In the earlier experiments the rats were allowed free access to food and to 0.9% sodium chloride solution. In Figure 1 are presented the data for sodium intake and sodium balance[3] for adrenalectomized and sham adrenalectomized rats subjected to fracture of the bones of both hind legs. A marked sodium retention (positive sodium balance) was exhibited by the adrenalectomized rats on the first post-fracture day. This was followed by a negative sodium balance on the second post-fracture day. Sodium intake, primarily in the form of the saline solution, was slightly reduced on the first post-fracture day and greatly reduced on the second day. There

[2] Whatman No. 1 filter paper was washed five times with 0.75N. nitric acid and five times with distilled water and then dried overnight at 105–110° C.

[3] The term "balance" as used here represents the difference between intake and urinary excretion.

was only a slight positive sodium balance in the sham adrenalectomized rats on the first post-fracture day. Sodium intake was not appreciably changed.

The potassium data for these experiments are presented in Figure 2. Following fracture, both the adrenalectomized and sham adrenalectomized rats exhibited a negative potassium balance. These results are complicated by the reduction in potassium intake by the adrenalectomized rats. Thus

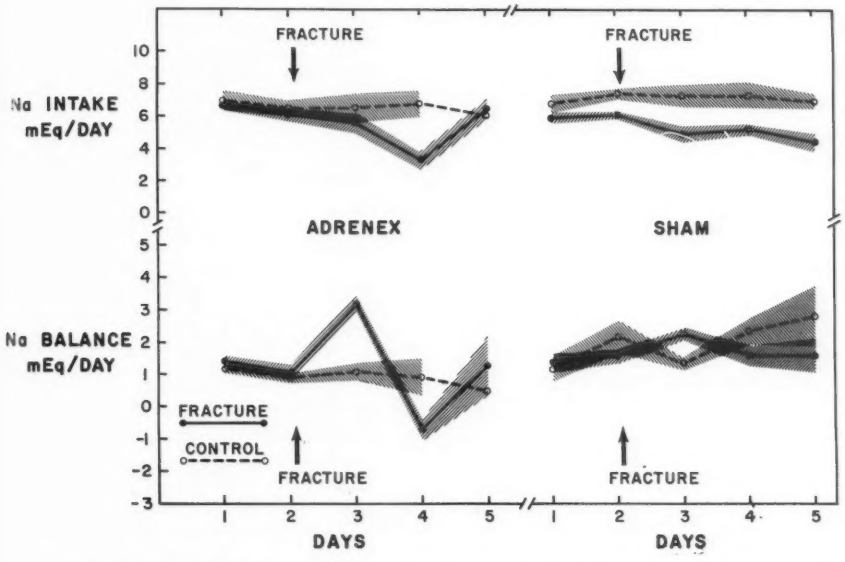

FIG. 1. The effect of hind leg fracture on sodium intake and sodium balance in adrenalectomized and sham adrenalectomized rats. The data for sodium intake are presented in the upper portion and for sodium balance (intake—urinary excretion) in the lower portion of the figure. The solid lines represent the mean data for the rats subjected to fracture, the dashed lines the mean data for the non-fractured controls. The shaded areas indicate ± one standard error of the mean.

it is seen that adrenalectomized rats maintained with supplemental sodium chloride show the sodium retention and potassium loss which have been classified as part of the typical response to injury. However, because of the variation in the intake of food and saline after fracture all subsequent experiments were carried out on force-fed rats.

On the first day after fracture, urinary sodium excretion was reduced in the force-fed adrenalectomized and the force-fed sham adrenalectomized rats, but to a much greater extent in the former (Figs. 3 and 4). On the second and third post-fracture days urinary sodium excretion in the adrenalectomized rats slightly exceeded control values.

Urinary potassium excretion in the adrenalectomized rats was slightly

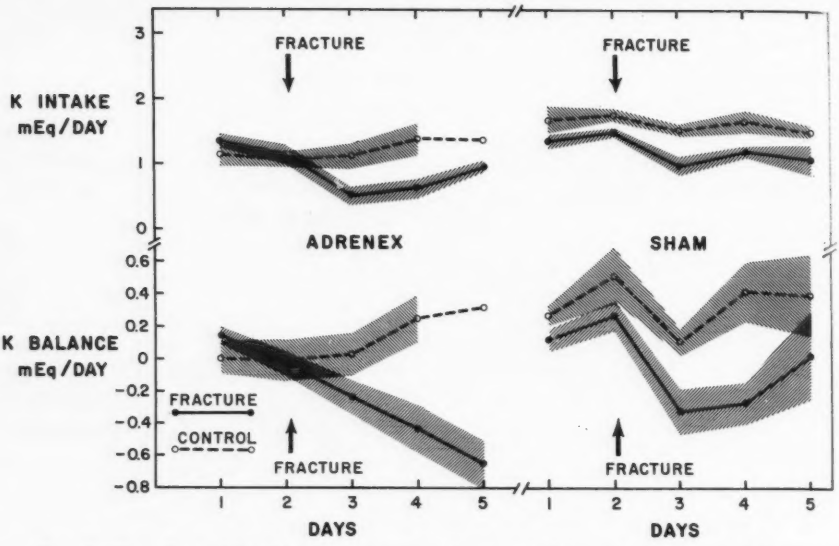

FIG. 2. The effect of hind leg fracture on potassium intake and potassium balance in adrenalectomized and sham adrenalectomized rats. See Figure 1 for the method of presentation.

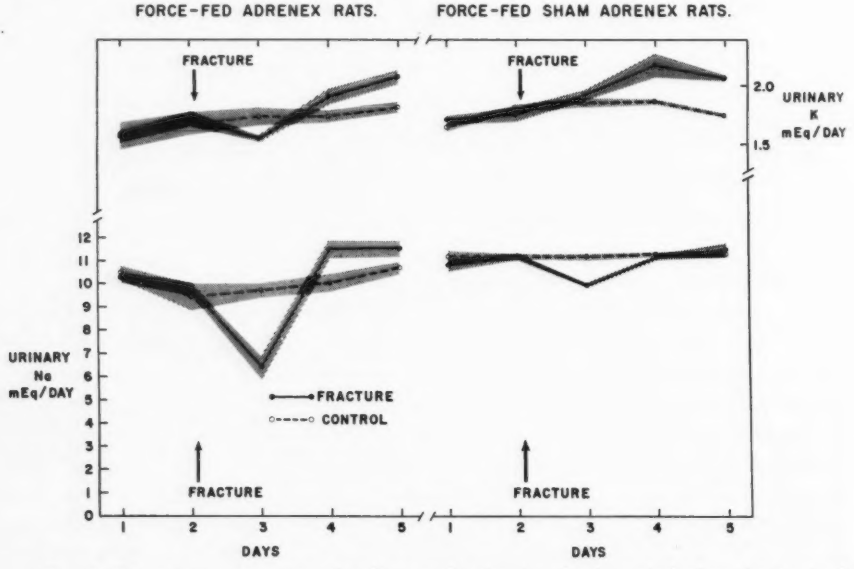

FIG. 3. The urinary excretion of sodium and potassium by force-fed adrenalectomized and sham adrenalectomized rats subjected to fracture. Urinary potassium excretion is presented in the upper portion of the figure and urinary sodium excretion in the lower portion. The solid lines represent the means for the fractured rats and the dashed lines the means for the non-fractured controls. The shaded areas indicate ± one standard error of the mean.

reduced on the first post-fracture day (Figs. 3 and 4). At this time potassium excretion in the sham adrenalectomized rats subjected to fracture was slightly increased (Figs. 3 and 4). By the second post-fracture day potassium excretion was markedly elevated in both groups of rats, and not return to control levels until the 6th to 8th post-fracture days. The total net post-fracture potassium loss was greater in the sham adrenalectomized rats than in the adrenalectomized rats. For the experiment summarized in Figure 4, the net potassium loss was 2.0 m.eq. for the sham adrenalectomized rats and 1.2 m.eq. for the adrenalectomized rats.

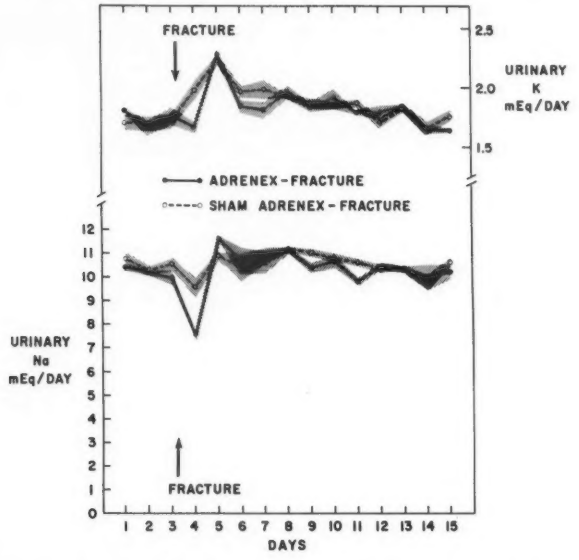

FIG. 4. The urinary excretion of sodium and potassium by force-fed adrenalectomized and sham adrenalectomized rats subjected to fracture. See Figure 3 for the method of presentation. In this experiment there were no non-fractured controls.

A pertinent question concerns the source of the excess potassium appearing in the urine of the injured adrenalectomized rat. In an attempt to determine whether the net loss of potassium could be accounted for solely by tissue damage the following experiment was performed. Six days after fracture adrenalectomized rats were sacrificed and the total sodium and potassium contents of the muscle and bone of both hind legs were determined for each rat. The potassium content of the muscle and bones of both hind legs is referred to as the total hind leg potassium content. These results are summarized in Tables 1 and 2. In order to estimate the amount of potassium lost from uninjured tissues following fracture, the increment over normal in urinary potassium excretion in the six day post-fracture period (post-fracture excess urinary potassium loss) was corrected for the loss of potassium from the injured hind legs and for a tendency for potas-

sium excretion to increase with time in the non-fractured rats. The details of these calculations follow:

(1) Potassium lost from uninjured tissues (Table 1, column 5-B) = post-fracture excess urinary potassium loss (Table 1, column 3-B) − correction for increased urinary potassium excretion in non-fractured rats (Table 1, column 3-A) − potassium lost from injured hind legs.

TABLE 1. POTASSIUM LOST FROM INJURED TISSUES AFTER FRACTURE

Rat No.	Mean pre-fracture urinary K, m.eq./day	Post-fracture excess urinary K loss, m.eq.	Total hind leg K, m.eq.	K lost from uninjured tissues, m.eq.
		Non-fractured adrenalectomized rats		
(*1A*)	(*2A*)	(*3A*)	(*4A*)	
1	1.69	0.48	1.94	
2	1.88	+0.43[1]	1.94	
3	1.74	0.01	2.22	
4	1.73	0.17	2.39	
5	1.66	—	2.27	
6	1.74	0.65	2.20	
7	1.76	1.07	2.12	
Mean ±S.E.	1.74 ±0.03	0.33 ±0.22	2.15 ±0.06	
		Fractured adrenalectomized rats		
(*1B*)	(*2B*)	(*3B*)	(*4B*)	(*5B*)
8	1.69	0.63	1.73	+0.12[3]
9	1.58	1.52	1.65	0.69
12	1.73	1.53	1.85	0.90
13	1.89	0.12	1.79	+0.57[3]
14	1.64	1.21[2]	2.04	0.77
15	1.55	1.44	2.15	1.11
16	1.70	1.06	2.27	0.85
17	1.73	1.69	2.02	1.23
Mean ±S.E.	1.69 ±0 12	1.15 ±0.19	1.94 ±0.08	0.61 ±0.21
	p>0.05	0.02>p>0.01	p=0.05	0.05>p>0.02

See text for details of preparation of table.

[1] Net K retention during this period.

[2] Urine for second post-fracture day (time of peak K excretion in fractured rats) lost; therefore value for K lost from uninjured tissues is probably underestimated.

[3] The data would appear to indicate that there was a gain of K by tissues other than the hind leg.

(2) Post-fracture excess urinary potassium loss (Table 1, column 3-B) = $\sum$ [daily potassium excretion in post-fracture period − mean daily urinary potassium excretion in pre-fracture period (Table 1, column 2-B)].

(3) Correction for increased urinary potassium excretion in non-fractured rats (Table 1, column 3-A) = $\sum$ [daily urinary potassium excretion in post-fracture period − mean daily urinary potassium excretion in pre-fracture period (Table 1, column 2-A)].

(4) Potassium lost from injured hind legs = mean potassium content of non-injured hind legs (Table 1, column 4-A) − potassium content of injured hind legs (Table 1, column 4-B).

On the basis of these calculations in six rats there is a loss of potassium from uninjured tissue which amounts to half or more of the post-fracture

excess urinary potassium loss. In two rats (Nos. 8 and 13) there appears to be a gain of potassium by uninjured tissue. The gain of sodium was greater than the loss of potassium in the hind leg muscle of all the fractured adrenalectomized rats (Table 2). A possible explanation for the gain in muscle sodium exceeding potassium loss is that there was an expansion of the extracellular space of the injured muscle. This is not precluded by the fact that the percentage water content of the hind leg muscle from fractured rats was essentially the same as that from non-fractured animals,

TABLE 2. WATER AND ELECTROLYTE CONTENT OF HIND LEG MUSCLE FROM FRACTURED AND NON-FRACTURED ADRENALECTOMIZED RATS

Rat No.	Water content %	Na		K		Body weight, gm.
		m.eq./kg. fresh muscle	m.eq./kg. F.F.D.S.[1]	m.eq./kg. fresh muscle	m.eq./kg. F.F.D.S.[1]	
		Non-fractured adrenalectomized rats				
1	74.7	18.4	84.7	105.2	483	235
2	74.7	17.7	81.3	105.6	485	244
3	74.0	19.5	88.0	107.3	484	272
4	73.1	18.4	81.4	109.8	487	274
5	74.3	18.6	83.5	107.1	481	271
6	72.5	17.8	82.0	102.3	479	275
7	73.7	24.2	108.4	100.3	450	296
Mean ±S.E.	73.9 ±0.35	19.2 ±1.5	87.0 ±9.2	105.4 ±1.6	478 ±3.8	—
		Fractured adrenalectomized rats				
8	76.3	41.9	217.7	81.5	423	248
9	73.0	30.5	151.0	88.8	439	232
10	73.4	31.6	150.0	91.3	433	233
11	75.2	28.4	132.0	94.1	435	228
12	72.6	24.1	110.0	98.0	448	274
13	74.2	26.7	121.7	98.7	451	262
14	73.5	28.1	131.8	94.3	442	283
15	74.9	29.4	138.7	87.7	414	273
16	74.0	28.3	144.1	88.3	450	264
17	73.7	28.3	133.7	90.9	430	268
Mean ±S.E.	74.1 ±0.31	29.7 ±0.9	143.1 ±3.7	91.4 ±1.2	437 ±4.8	—
	$p > 0.05$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	

[1] Fat-free dry solids.

since there might have been a loss of water from injured cells. Separate bone electrolyte analyses are not presented because it was not possible to completely remove all of the muscle from the fractured bones.

The marked sodium retention which occurred on the first post-fracture day in the adrenalectomized rats had a profound effect on water balance. Coincident with the sodium retention there was a reduction in urine volume and an increased intake of water. The sham adrenalectomized rats subjected to fracture showed only a moderate retention of sodium, and in these animals there were no apparent changes in either urine volume or water intake.

The concentrations of sodium and potassium in the plasma of adrenalectomized and sham adrenalectomized rats were determined at various time intervals after fracture (Table 3). Bone fracture induced a significant in-

TABLE 3. EFFECT OF HIND LEG FRACTURE ON PLASMA Na AND K CONCENTRATIONS IN FORCE-FED RATS

Hrs. post-fracture	No. of rats	Na	K
		(m.eq./liter (mean ±S.E.)	
		Adrenalectomized	
Control	22	143 ±1.1	4.86 ±0.14
24	11	146 ±2.0	5.22 ±0.19
48	6	144 ±1.9	5.62 ±0.16
72	5	138 ±1.4	5.20 ±0.20
		Sham-adrenalectomized	
Control	26	146 ±0.9	3.73 ±0.06
2½	10	146 ±1.0	4.38 ±0.11
24	15	141 ±0.7	4.23 ±0.13
48	10	142 ±0.6	4.12 ±0.09
72	9	148 ±0.9	3.77 ±0.12

crease in plasma potassium concentration, with a peak on the second post-fracture day in the adrenalectomized rats and 2½ hours after fracture in the sham adrenalectomized rats.

DISCUSSION

The data which have been presented demonstrate that potassium loss and sodium retention follow severe tissue injury in the rat in the absence of the adrenal cortex. These findings are in accord with those of Noble and Toby (9) that the adrenal cortex is not required for the protein catabolism which follows trauma.

Let us first consider the post-traumatic changes in potassium metabolism. The data indicate that the loss of potassium from injured tissues can only partially account for the excess potassium lost in the urine following bone fracture in adrenalectomized rats. It must be concluded, then, that potassium has been lost from cells remote from the site of injury in these animals. This may be the result of either of two mechanisms: Following trauma, a factor other than adrenocortical secretion (a) causes an increased renal excretion of potassium, secondarily depleting the cells of potassium, or (b) directly induces the release of potassium from cells which have not been physically injured. The first of these possibilities seems unlikely. The elevated plasma potassium concentration in the first several days after fracture would suggest that the role of the kidney is secondary, excreting potassium which has been liberated from extrarenal sites. What might be the nature of such a factor which brings about the loss of potassium from uninjured cells following tissue trauma? At the present time we know of no hormonal (other than adrenocortical) or neural factors which could induce the observed effects. Therefore, we suggest that potassium is lost from uninjured cells as a result of the direct action of a "toxic factor" which is formed at the site of tissue damage. The quantity of potassium which we have estimated is lost from uninjured tissues fol-

lowing fracture in the adrenalectomized rat is relatively small as compared to the total intracellular potassium pool. Consequently, the physiological significance of this loss is questionable. However, following bone fracture in the rat there is an increased adrenocortical activity (10), which may accentuate the post-traumatic negative potassium balance as evidenced by the greater loss of potassium in the fractured sham adrenalectomized as compared to the fractured adrenalectomized rats.

The observation that the adrenalectomized rats retained more sodium following fracture than did the sham adrenalectomized rats is at first sight a paradoxical situation, since one of the outstanding defects of adrenal insufficiency is an impaired ability to retain sodium. However, these rats were on a very high sodium intake, and it has been previously demonstrated that animals in adrenal insufficiency are unable to handle a salt load primarily as a consequence of a reduced glomerular filtration rate (17). It would seem to be a reasonable assumption that the adrenalectomized rats were in a state of adrenal insufficiency in the immediate period after fracture, with a consequent reduction in the glomerular filtration rate. They would then be unable to excrete adequately the large amount of dietary salt. The relative roles of a possible reduction in glomerular filtration rate and an increased secretion by the adrenal cortex in the sodium retention found in the sham adrenalectomized rats cannot be assessed at this time.

There have been attempts to determine by direct analysis the effects of trauma on the electrolyte content of untraumatized tissues. MacPhee (18), using rats, found a small loss of cellular potassium and a gain of cellular sodium in the untraumatized hind leg muscle 2 to 20 hours after removal of a tourniquet from the opposite leg (thiocyanate was used to estimate extracellular space). These changes did not occur in adrenalectomized rats. Fox and Baer (19), using mice, describe what appear to be opposite results: a gain of potassium and a loss of sodium from one hind leg 4 to 6 hours after removal of a tourniquet from the opposite leg, or when the opposite leg was scalded. These authors analyzed the whole hind leg for sodium and potassium and did not attempt to separate intracellular from extracellular electrolyte. One must be careful in interpreting the data. The total amount of "excess" potassium lost in the urine following trauma is relatively small compared to the total intracellular potassium content. Consequently, the loss of potassium from non-injured tissue is relatively small. In order to measure small changes in intracellular potassium concentration it is necessary to be able to accurately partition the total tissue potassium into its extracellular and intracellular components. Unfortunately a high degree of accuracy is not possible with current techniques.

The decreased output of urine by the adrenalectomized rats on the first day after fracture was most likely a consequence of the marked retention of sodium, since the reabsorption of water by the kidney is in large part passively linked to sodium reabsorption. The increased water intake by the

adrenalectomized rats was probably also a result of the sodium retention.

Although the changes in the metabolism of sodium, potassium and nitrogen, which in part characterize the individual's response to severe tissue injury, can occur in the absence of the adrenal, the secretions of the adrenal cortex reduce mortality following fracture. Approximately one-third of the adrenalectomized rats died within 24 hours after being subjected to the fracture procedure; none of the sham adrenalectomized rats died as a result of bone fracture. These observations are in accord with the well established fact that the adrenalectomized animal is hypersensitive to trauma.

In contrast to this, it is of interest to note the recent report by McKenna and Zweifach (20) that very large doses of cortisone or Meticorten impair the ability of rats to survive the trauma of rotation in a Noble-Collip drum. Thus, the physiological role of adrenocortical hormones in situations of trauma and stress still remains obscure.

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THE DEVELOPMENT OF AN ANEMIA IN RATS AFTER THYROIDECTOMY AT BIRTH

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ABSTRACT

New born rats were thyroidectomized by the intraperitoneal injection of I^{131} and maintained on a low iodine diet. There was a cessation of body weight increase after the age of 3 weeks. The normal rise in erythrocyte count following birth stopped after 3 weeks. At 8 weeks of age the thyroidectomized animals had a RBC count 78% of that of the normal controls. Hematocrit and hemoglobin concentration also failed to rise after 3 weeks of age and at two months were 79% and 87% respectively of the normal values. At 3 months the value fell further to 59% and 74% of the respective normal levels The bone marrow was aplastic. The relative weight of the spleen (mg/100 gm body weight) was decreased in the experimental animals and showed some atrophic changes with loss of germinal centers. Thus thyroidectomy of the newborn rat results in a progressive hyperchromic-microcytic anemia and the relationship of this anemia to that following hypophysectomy is discussed.

GOLDBERG and Chaikoff described a simple procedure for thyroidectomy of the newborn rat by the use of radioactive iodine (1). During our investigations of the metabolic effect of radiothyroidectomy at birth, we observed changes in the blood picture not mentioned by authors dealing with the effect of thyroidectomy (by various methods) of newborn rats (2, 3). This paper reports profound changes in the blood and erythropoietic tissues developing after radiothyroidectomy at birth.

MATERIAL AND METHODS

The animals used in these experiments were albino rats from a random bred colony. Radiothyroidectomy was performed according to the method of Goldberg and Chaikoff (1) by intraperitoneal injection of 100–120 μc. per rat of I^{131} in 0.1 cc. saline during the first 24 hours after birth. The injected litter mates and the mother received a low iodine test diet (Nutritional Biochemicals Co., Cleveland, Ohio) and distilled water *ad lib.* Only completely thyroidectomized animals were used. The thyroid was considered totally destroyed when growth ceased three weeks after the injection, and when histological proof of the absence of thyroid tissue was obtained in serial sections. Control animals were fed purina chow checkers (Ralston Purina Co., St. Louis, Mo.) and received tap water *ad lib.* Radio-thyroidectomized rats which were given a local rat diet consisting of sprouted wheat, barley, bran, skim milk powder and seasonal vegetables, developed the same anemia.

Since no change in the blood picture was found within the first week following administration of I^{131}, examinations were carried out two and three weeks, one month, two

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months and three months following the I^{131} injection. Blood samples were taken, when possible, from the tail. (When the animals were too small, the blood was taken by cardiac puncture and the animals discarded.)

Erythrocyte counts were done in duplicates using US certified blood pipettes and the improved Neubauer Counting Chamber.

Hemoglobin determinations were made by the alkaline hematine method according to Dacie (4).

Hematocrit was done according to Van Allen (5). Fragility test was made by the method of Haden (6).

The bone marrow was taken from the femur. Touch preparations from fresh bone marrow were stained with the May-Grünwald-Giemsa technique. Bone marrow was fixed in Helly's fluid and embedded in paraffin. Sections were stained with hematoxylin-eosin. The erythyroid-myeloid ratio was determined by differential counts on touch preparations. The spleen was also examined histologically in touch preparations and in paraffin sections.

RESULTS

The results are summarized in Table 1. Retardation of growth in the injected animals was already seen one week after the injection (findings not included in the table) and at the age of three weeks complete cessation of growth was observed. The animals had a small skeleton, dry skin, scaly tail, ruffled hair, and were still nursing when two months old.

During the first two weeks the amount of damage in the thyroid was different in various animals. Two to three weeks after the injection the follicular arrangment of the thyroid had in most cases disappeared, and in later stages the specific parenchyma was in all cases replaced by fat and connective tissue.

In normal suckling rats a continuous rise in the number of erythrocytes per $mm.^3$ was seen during the first weeks of life. A similar increase was observed in the radiothyroidectomized animals until the age of three weeks only. The level reached at this age was maintained thereafter, while the erythrocyte count in the normal animals continued to rise until the age of eight weeks. At this age, the radiothyroidectomized animals showed a count of only 78% of the normal values.

The hematocrit and hemoglobin in the radio-thyroidectomized animals showed a slight initial rise until three weeks, parallel to the rise in erythrocyte count. They remained at this three week level till two months, at which time they were respectively 79% and 87% of the normal values. At three months there was a considerable decrease in both these values to 59% and 74% of the respective normal values. The calculated mean corpuscular volume of the erythrocytes also showed a decrease at three months. The hemoglobin content of erythrocytes remained approximately normal during the whole time of the experiment, and this explains the increase in the corpuscular hemoglobin concentration.

After four weeks there was a more rapid decrease in the reticulocyte counts of treated animals towards the adult level than in normal animals.

TABLE 1. THE EFFECT OF RADIO-THYROIDECTOMY OF THE NEWBORN RAT ON THE HEMATOLOGICAL VALUES IN VARIOUS AGE GROUPS AS COMPARED WITH THOSE OF NORMAL RATS OF THE SAME AGE GROUP

Group*	Age	No. of rats	Body weight, gm.	Erythrocytes $\times 10^6$ per cu. mm.	Hematocrit %	Hemoglobin, gm./100 cc.	M.c.v.[1] μ^3	M.c.h.[2] μgm.	M.c.h.c.[3] %	Reticulocytes %	Spleen weight mg./100 gm. body weight %	Erythroid/myeloid ratio
N	2 weeks	5	24.0± 6.0	4.67±0.16	36.0±1.2	11.9±0.2	77.1	25.5	33.1	28.5±1.1	0.43±0.05	2.63±0.15
R		6	14.7± 0.5	4.82±0.23	37.9±1.2	12.7±0.3	78.6	26.3	33.5	23.0±3.6	0.21±0.02	2.22±0.14
N	3 weeks	9	34.7± 1.4	5.28±0.21	36.9±1.0	12.2±0.3	79.9	23.1	33.1	12.3±0.8	0.43±0.04	2.27±0.13
R		5	17.3± 1.3	6.20±1.01	39.9±0.9	13.2±0.2	63.8	20.0	33.1	4.8±0.9	0.21±0.02	1.12±0.03
N	4 weeks	7	60.8± 4.5	6.17±0.05	40.0±0.7	12.3±0.2	64.8	19.9	30.1	7.5±1.6	0.40±0.06	1.71±0.15
R		6	18.5± 1.3	6.51±0.35	40.0±1.2	13.0±0.4	61.4	20.0	32.5	2.9±0.6	0.24±0.03	1.00±0.09
N	8 weeks	17	160.0± 9.0	8.73±0.15	50.0±0.7	15.2±0.2	57.3	17.4	30.4	2.0±0.4	0.30±0.02	0.73±0.12
R		4	25.0± 2.5	6.80±0.17	39.5±2.3	13.3±0.2	58.0	19.0	33.6	2.8±1.3	0.14±0.02	1.08±0.18
N	12 weeks	17	203.0±22.0	8.73±0.15	50.0±0.7	15.2±0.2	57.3	17.4	30.4	2.0±0.4	0.25±0.02	0.73±0.12
R		9	30.0± 1.4	6.50±0.07	29.7±0.2	11.3±0.2	54.7	17.4	38.0	1.3±0.5	0.13±0.01	0.91±0.06

± Standard error $\sqrt{\Sigma d^2/n(n-1)}$.
* N—normal rats.
R—radio-thyroidectomized rats.
[1] Mean corpuscular volume.
[2] Mean corpuscular hemoglobin.
[3] Mean corpuscular hemoglobin concentration.

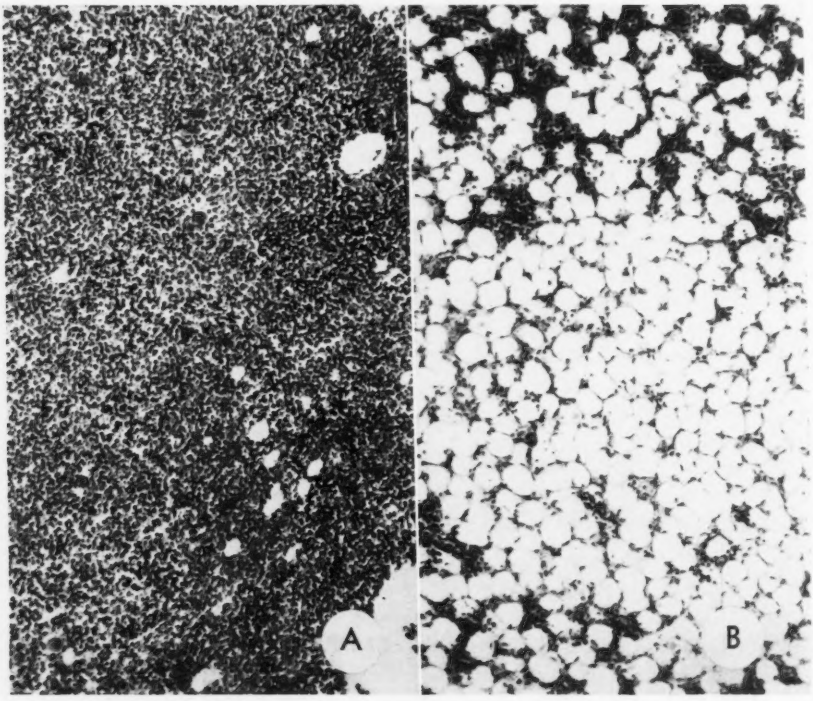

FIG. 1. Bone marrow section of three months old normal (A) and radiothyroidectomized (B) rats. H. and E. ×105.

No changes were found in the fragility of the erythrocytes. After two weeks the relative weight of the spleen (mg./100 gm. body wt.) decreased in the treated animals. At the age of twelve weeks the spleen was about 50% of the normal relative weight. Microscopically no germinal centers were seen in the malpighian follicles. The pulp was rich in macrophages and yellow pigment, contained only small amounts of blood, and showed fibrosis.

In the radiothyroidectomized rats the bone marrow showed decreased cellularity from three weeks on, and by the age of three months the areas of fatty tissues exceeded those of erythropoietic (Fig. 1). The erythroid-myeloid ratio was normal at all ages except at three weeks, when it was reduced.

DISCUSSION

It was found in these experiments that radiothyroidectomy in the rat on the first day of life caused a moderate anemia to appear two months after the administration of I^{131}. By three months hematocrit and hemoglobin values had fallen progressively, but the number of erythrocytes did not change from the two month value. This anemia was accompanied by

a severe aplasia of the bone marrow and a considerable decrease in the weight of the spleen. It was different in degree and type from that seen following surgical thyroidectomy (as well as in clinical hypothyroidism) in adults of various species, which (according to most investigators) is of a moderate degree and generally normocytic-normochromic (7, 8, 9).

Kunde, Green and Burns (10) reported a severe macrocytic-hyperchromic anemia five weeks after surgical thyroidectomy of 2–3 week old rabbits. Their findings differ from ours only with respect to corpuscular volume. The development of severe and progressive anemia in our experiments and in those of Kunde et al. differs from the mild anemia in animals thyroidectomized at later ages and shows that the age at which thyroidectomy is performed apparently determines the type of anemia. Hughes (11) treated 27 rats with thiouracil from birth and found a slight anemia in 26, and a severe anemia in one animal only. The anemia obtained in our experiments closely resembled that found after hypophysectomy in its degree, rate of progress and mean corpuscular volume (7, 8, 12–17).

The red count in normal animals showed a steady increase from birth to maturity. As known from other investigations (18), and confirmed in our experiments, cessation of body growth in rats occurs three weeks after thyroidectomy and there is subsequently no increase in red cell count. The level reached at three weeks was maintained throughout the whole observation period, and at three months was 74% of the normal value. This indicates that the red count in these animals is an expression of their body weight and metabolic state and not of their age.

The hematological picture changed progressively in the direction of fall in hemoglobin and hematocrit. This indicates that radiothyroidectomy at birth influences not only erythropoiesis but also the formation of hemoglobin. The severe bone marrow aplasia, with replacement of red marrow by fat, resembles that described after hypophysectomy and differs from the slight aplasia following surgical thyroidectomy in adult animals (14, 16).

We have found no data in the literature on changes in weight and histology of the spleen after thyroidectomy. After hypophysectomy a decrease in the weight of the spleen has been recorded (17, 19). In our rat strain permanent extramedullary hematopoiesis occurred in the spleen (20), and it is possible that the atrophy of that organ contributed to the anemia. It remains to be determined whether the above described results are caused directly by the absence of thyroid hormone or whether they are mediated through the pituitary, as suggested by Eartly and Leblond (21).

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We wish to thank Prof. A. Rosin for her advice and interest during our work on this problem.

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STEROIDS IN THE BLOOD AND URINE OF FEMALE MICE BEARING AN ACTH-PRODUCING PITUITARY TUMOR[1]

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ABSTRACT

It has been reported that female LAF_1 mice bearing an ACTH-producing tumor exhibit symptoms somewhat resembling those of Cushing's disease in humans (1). The blood serum and urine of these mice have now been analyzed for steroid compounds in an effort to ascertain the nature of the adrenocortical secretion.

Cortisol and its metabolites retaining the phenylhydrazine reacting side chain were present only in traces or absent in both serum and urine, despite the prolonged action of endogenous ACTH. Corticosterone appeared to be the major corticoid of serum, while in urine the most abundant C_{21} steroid could have been a metabolite of corticosterone.

The concentration of C_{19} steroids in serum was roughly equal to that of corticosterone, whereas in urine it was forty times the corticoid total. The predominating urinary steroids were $C_{19}O_3$ compounds. Two constituents of this type which were isolated were most probably 11β-hydroxy-Δ^4-androstene-3,17-dione and its saturated analogue 11β-hydroxy-androstane (or etiocholane)-3, 17-dione. Unknown $C_{19}O_2$ steroids were also present in urine, but neither dehydroepiandrosterone, androsterone, nor etiocholanolone was found.

Thus 11β-hydroxy-Δ^4-androstene-3,17-dione was probably the major adrenal product; corticosterone and possibly other steroids were secreted to a lesser extent.

PRONOUNCED physiological effects of hyperadrenalcorticism produced in mice by transplanted ACTH secreting tumors have been described (1). In an effort to relate the physiological findings to actual adrenocortical steroids produced by these mice, their blood and urine have been analyzed for steroid compounds.[3] This communication describes these experiments.

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[3] The following "trivial" names will be used for the various steroid compounds:
Androstanedione is androstane-3,17-dione.
Etiocholanedione is etiocholane-3,17-dione.
Androsterone is 3α-hydroxyandrostane-17-one.
Dehydroepiandrosterone (DHA) is 3β-hydroxy-Δ^5-androstene-17-one.
Testosterone is 17β-hydroxy-Δ^4-androstene-3-one.

(*Continued on next page*)

Evidence from the previous work of other investigators on the routes of excretion of steroid hormone metabolites in mice, led to the expectation that amounts sufficient for isolation could be recovered from the urine. Barry *et al.* (2) found 30 to 50% of the radioactivity derived from intraperitoneally administered testosterone-4-C^{14} in the urine of mice. However, only 35% of this (or about 15% of the total dose) could be extracted with ether after hydrolytic procedures which give much higher recoveries from the urine of human subjects (3). The authors conclude that in mice the principal metabolites of testosterone are not androsterone and etiocholanolone as is the case in humans. The disposition of an adrenocortical steroid in mice has been followed by Bradlow *et al.* (4), who gave cortisone-T. After intravenous injection, 65% of the radioactivity appeared in the urine in 24 hours. Deoxycorticosterone-21-C^{14} acetate was given subcutaneously to mice by Heard *et al.* (5), with recovery of approximately 30% of the radioactivity in the urine after 5 days, of which about 44% was extractable by ether after various hydrolytic procedures.

In the present study, female LAF_1 mice stimulated with endogenous ACTH, secreted steroidal precursors whose metabolites could be extracted from urine in substantial amounts after several hydrolytic procedures. Ketosteroids of $C_{19}O_3$ structure comprised by far the largest portion of the metabolites. Two of these are most probably 11β-hydroxy-Δ^4-androstene-3,17-dione, (11-OH4AD) and 11β-hydroxy-androstane (or etiocholane)-3,17-dione, and others have been partially characterized. Smaller amounts of $C_{19}O_2$ steroids have also been found, but none could be identified. The chief C_{21} compound in urine was not corticosterone, but possibly one of its metabolites. The blood plasma of both tumor bearing and control mice yielded a substance which was probably corticosterone.

METHODS

Reagents and techniques were as previously described (6) with the following additions:

Solvents: *n-Hexane:* Purified by washing with $\frac{1}{5}$ volume of concentrated sulfuric acid until no more yellow color is removed, then washing with water until neutral. After drying over anhydrous sodium sulfate, the filtered hexane is redistilled through a Vigreaux column.

Ligroin: Eastman Kodak #P-1628. Purified exactly as for n-hexane. On redistillation, the fraction boiling from 103–106° is collected.

11-OH4AD is 11β-hydroxy-Δ^4-androstene-3,17-dione.
Deoxycorticosterone (DOC) is 21-hydroxy-Δ^4-pregnene-3,20-dione.
Corticosterone (Cpd. B) is 11β,21-dihydroxy-Δ^4-pregnene-3,20-dione.
Substance S is 17α,21-dihydroxy-Δ^4-pregnene-3,20-dione.
Tetrahydrocorticosterone is 3α,11β,21-trihydroxypregnane-20-one.
Cortisone (Cpd. E) is 17α,21-dihydroxy-Δ^4-pregnene-3,11,20-trione.
Cortisol (Cpd. F) is 17α,11β,21-trihydroxy-Δ^4-pregnene-3,20-dione.
Tetrahydrocortisone is 3α,17α,21-trihydroxypregnane-11,20-dione.
Tetrahydrocortisol is 3α,11β,17α, 21-tetrahydroxypregnane-20-one.

Paper chromatography by the method of Bush (7): All operations were carried out at a controlled room temperature which remained fairly constant at 24° C. The chromatography tanks were saturated for at least one day before use, by placing stationary phase in the bottom and a liner of Whatman #3 MM paper around the sides. Four "wicks" dipping into 2 beakers of stationary and 2 of mobile phase also extended to the top of the tank. After application of the material to be chromatographed, the papers were equilibrated in the tank for 16 hours before development with mobile phase.

Modified Silber-Porter Assay: Reagents were prepared as described by the authors (8). Two equal samples of the material to be assayed were dried in 10×75 mm. tubes and redissolved in 0.5 ml. of chloroform. One-half ml. of the ethanolic sulfuric acid reagent was added to one tube and one-half ml. of phenylhydrazine reagent to the other and the contents were thoroughly mixed with a footed glass rod. After 24 hours of color development at room temperature, the reagent layer (upper) was read at 410 mμ on a Beckman Model DU Spectrophotometer equipped with micro cells.

PROCEDURES AND RESULTS

I. Steroids in Blood Serum:

Serum from both intact control mice and tumor-bearing mice was analyzed. Blood was obtained by decapitation of 20 control mice and 30 mice bearing tumors. Each sample of pooled blood was centrifuged yielding 10 ml. serum from control animals and 16 ml. from the tumor-bearing animals.

Enzyme hydrolysis: Each serum sample was adjusted to pH 5.0 with approximately 1 ml. of 1N sulfuric acid. Beef liver β-glucuronidase ("Ketodase," Warner-Chilcott) was added, using 500 units per ml. of original serum. After adding a few ml. of chloroform, the mixture was incubated at 38° C. for 24 hours.

Extraction of fat: One and one-half volumes of absolute ethanol were added to the clear incubated plasma, making a concentration of 60% ethanol. The plasma proteins precipitated leaving a clear supernatant. These mixtures were then well shaken in order to extract all lipid material into the 60% ethanol. Each entire mixture was then extracted four times with an equal volume of n-hexane. The combined hexane extracts were back-washed once with 10 ml. 60% ethanol and then discarded.

Extraction of steroids: The ethanol concentration of each mixture was reduced to 40% by addition of water, and 3 extractions with an equal volume of chloroform were performed, followed by one extraction with ethyl acetate. These combined solvents were set aside (Extract A).

Hot acid hydrolysis: Each entire remaining suspension of plasma proteins was adjusted to pH 1.0 with sulfuric acid, and then made to a final concentration of 5% sulfuric acid. It was then transferred to open Pyrex bottles and heated in boiling water for 20 minutes after the contents attained a temperature of 90°. After cooling, the mixtures were extracted 3 times with an equal volume of chloroform (Extract B).

Preparation of neutral fraction: Extracts A and B were combined and washed once with $\frac{1}{10}$ volume of an aqueous 10% Na_2CO_3 solution and once with $\frac{1}{6}$ volume of a solution of 0.02N acetic acid in 25% w/v aqueous sodium chloride. The washing procedure was designed to minimize losses of water soluble steroids. The remaining chloroform was evaporated to dryness *in vacuo*.

Removal of remaining lipids and other contaminants: The neutral extract fraction was then put through a "reverse-phase" column of Hyflo Super-Cel (Johns-Manville) treated with dimethyldichlorosilane. The procedure described by Morris *et al.* (9) was followed exactly. This technique has since been found too difficult for routine use. Moreover, polar, water soluble contaminants were eluted together with the steroids. These

were removed by partition of the dried eluate between 0.5 ml. H_2O and 3 ml. of a 3:1 mixture of ethyl acetate and ether.

Chromatography: The final extracts were chromatographed on paper in the Bush B 4 system (toluene:methanol:water, 100:50:50) (7). The extracts were applied to separate limbs of washed Whatman #1 paper 0.6 cm. wide, originating from the same head (6). Two additional limbs contained 5 and 10 μg., respectively, of both cortisol and corticosterone as reference standards. After development, two strips, each 0.15 cm. wide, were cut from each paper and submitted to the Bush "soda-fluorescence" test (10), and the triphenyl tetrazolium chloride reduction test (TPT).

Results on chromatograms of plasma extracts: Figure 1 shows the results of spot tests on the chromatograms. The serum from control mice showed no definite reducing areas, but three regions reacted to the more sensitive soda-fluorescence test for Δ^4-3-ketones. The first was near the origin, the second below the cortisol standard and hence probably not due to cortisol and the third was directly opposite the corticosterone standard.

The extract of serum from tumor mice gave much stronger reactions to both tests. Strong bands were seen just below the origin, but again there was

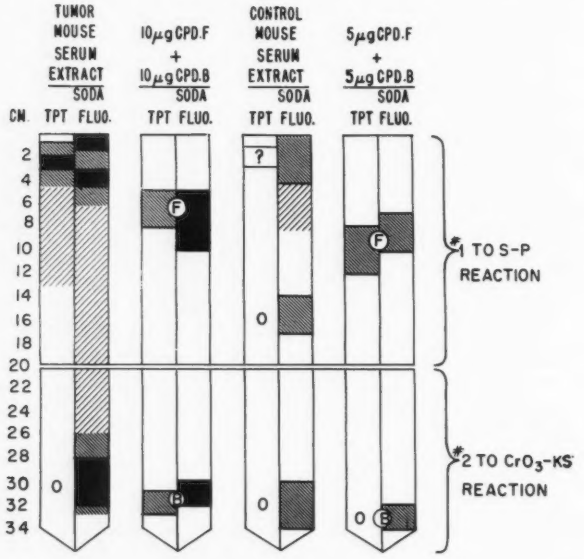

FIG. 1. Chromatograms of extracts of mouse serum and of reference steroids.
Solvent system: Toluene:methanol:water, 100:50:50. (Bush B4).
Time: 2 hours.
Temperature: 24° C.
Reference standards: Cortisol (Cpd. F) Corticosterone (Cpd. B).
Strip width—0.6 cm. total; 0.15 cm. used for each test.
TPT—Reduction test with triphenyltetrazolium chloride.
Soda fluo.—Soda fluorescence test for Δ^4-3-ketones.
Black or shaded areas indicate strong or weak tests respectively.

no localized reaction directly opposite cortisol. The region between the cortisol and corticosterone standards exhibited a continuous soda-fluorescence reaction, indicating that this paper may have been overloaded. A strong soda-fluorescence reaction was seen directly opposite corticosterone. Though the less sensitive TPT test was negative, this band could have contained corticosterone.

Semi-quantitative assays: The remaining 0.3 cm. of each paper was cut transversely into two sections and eluted. (See Fig. 1.) The more polar regions (#1 cuts), when tested by the modified Silber-Porter reaction, developed typical yellow colors equivalent to about 1 μg. cortisone per 10 ml. in the control serum and roughly twice this concentration in the tumor serum.

The less polar eluates (#2 cuts) were assayed by the CrO_3-KS reaction which determines almost all C_{21} and C_{19} 17-hydroxy steroids, as well as 17-ketosteroids (11). Both control and tumor sera gave characteristic purple colors corresponding to 6 μg. androsterone equivalent per 10 ml. The least polar material, which had run off the chromatograms during 2 hours, was also subjected to CrO_3-KS assay, showing 3 μg. and 5 μg. per 10 ml. serum in the control and tumor sera, respectively.

Conclusions from blood serum analyses: Table 1 summarizes the quantitative deductions from these assays. Although the diffuse spot tests near the origin appeared largely due to non-steroidal material, small amounts of highly polar 17,21-dihydroxy-20-ketones were suggested by the Silber-Porter assay. The Δ^4-3-ketone whose mobility coincided with that of corticosterone is assumed to be corticosterone, although the evidence is scant and not entirely consistent. Its amount was about 5 times that of the more polar constituents, making it the predominant corticosteroid. Both these

TABLE 1. QUANTITATIVE ANALYSES[1] OF STEROIDS IN CHROMATOGRAPHIC FRACTION OF MOUSE SERUM EXTRACTS

Type of steroid	Mobility in the Bush B4 system	How assayed[2]	Mouse serum content		
			Control, μg/10 ml.	Tumor, μg/10 ml.	Ratio: tumor / control
17,21-di-OH 20-ketones	Chiefly more polar than F	Silber-Porter reaction on Cut #1	1.0	2.0	2.0
Corticosterone	Same as Cpd. B	Estimated from localized soda-fluorescence spot test	5.0	10.0	2.0
17-Hydroxy- or 17-Ketosteroids	Near Cpd. B	CrO_3-KS reaction on Cut #2	6.0	6.0	1.0
17-Hydroxy- or 17-Ketosteroids	Less polar than Cpd. B	CrO_3-KS reaction on Runoff	3.0	5.0	1.7

[1] All values are subject to the limitations of estimations from spot tests on the papergrams and from colorimetry at very low concentrations, and are approximate only.
[2] C.f. Figure 1 for origin of material assayed.
See text for reactants in the CrO_3-KS determination.

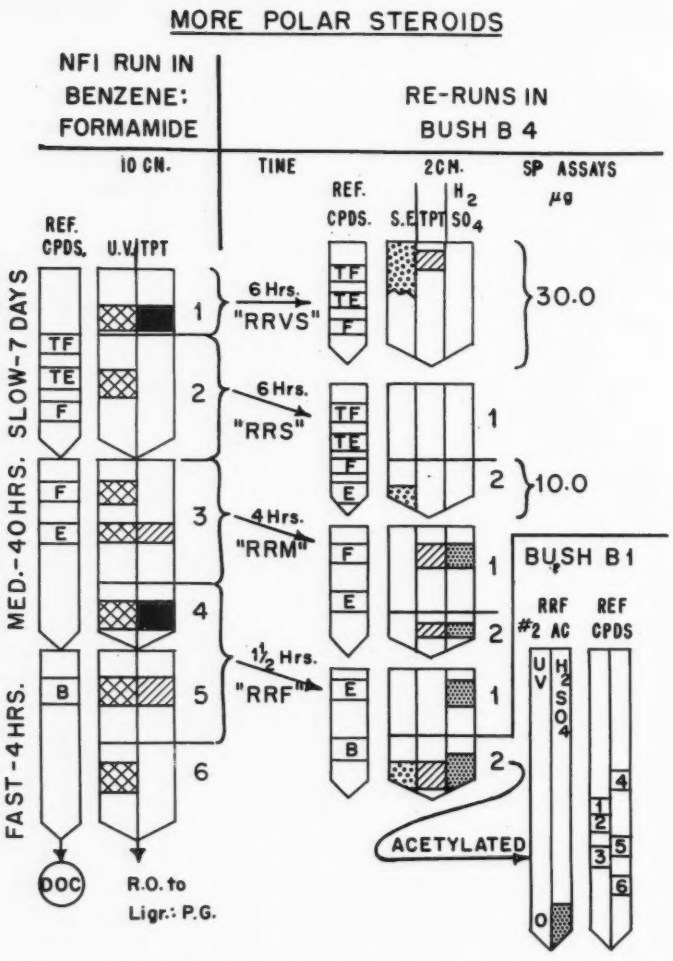

FIG. 2. Chromatograms of the more polar steroids in extracts of mouse urine after hydrolysis with β-glucuronidase and continuous ether extraction at pH 0.8 (NF 1).

U.V.—Absorption of ultra violet light.

TPT—Reduction of triphenyltetrazolium chloride. Black or shaded areas indicate strong or weak tests respectively.

H_2SO_4—Fluorescence under long wave ultra violet light after immersion in concentrated sulfuric acid.

S.F.—Soda-fluorescence test for Δ^4-3-ketones.

S-P—Quantitative Silber-Porter assays.

TF—Tetrahydrocortisol, TE-tetrahydrocortisone.

The numbers on the lower right hand chromatogram indicate the location of the following reference steroids, visualized by appropriate spot tests:

1. Cpd S acetate
2. Cpd. B acetate

types of corticosteroids were approximately doubled in concentration by the ACTH producing tumor.

The positive CrO_3-KS assays on eluates of the corticosterone region show that polar $C_{19}O_3$ steroids were most probably also present, and in amounts comparable to that of corticosterone itself. C_{19} compounds, such as 3,11,17-triols, 3,11-dihydroxy-17-ketones and 11β-hydroxy-testosterone, have mobilities similar to or less than that of corticosterone. This type of steroid was not detectably increased by the ACTH producing tumor. Less polar CrO_3-KS reactants, which could consist of faster moving $C_{19}O_3$ or $C_{19}O_2$ components, were almost doubled by the tumor.

These findings point strongly to 11-oxygenated C_{19} steroids as major components of mouse serum. Analyses of rat adrenal vein blood (12) have previously suggested the presence of 11β-hydroxy-Δ^4-androstene 3,17-dione in amounts up to 40% of the total steroids. Moreover, our results parallel those on rat adrenal vein blood in the predominance of corticosterone over more polar corticosteroids.

II. Steroids in Urine:

Collection of urine: Three mice were kept in metabolism cages for a period of 2 months after their transplanted tumor had become well established. A total of 150 "mouse days" of urine, at pH 8.9, had been stored in the cold for 1 year before being worked up. The total volume was 1.8 liters.

Hydrolysis of conjugates and extraction: Free steroids, glucuronides and readily hydrolyzed sulfates (NF 1): The urine was adjusted to pH 5.0, buffered, and 720,000 units of β-glucuronidase ("Ketodase") were added, making a concentration of 400 units per ml. After 72 hours incubation (in the presence of 50 ml. chloroform) it was acidified to pH 0.8 and continuously extracted with ether for 48 hours, changing the ether in the boiling flask each morning and evening. The total ether extract was washed twice with $\frac{1}{10}$ volume of 1.0N NaOH and once with $\frac{1}{10}$ volume of 0.02N acetic acid in 25% NaCl solution, giving the first neutral fraction, NF 1.

Partition of NF 1 between 70% Ethanol and Hexane: The dark brown, oily extract was dissolved in 100 ml. of 70% ethanol and extracted twice with 50 ml. of hexane, which was discarded. The ethanol concentration was reduced to 40% by adding water, and this solution was extracted 3 times with 75 ml. of chloroform. Subsequent discovery of both highly polar and least polar steroids indicates that this procedure was not responsible for any serious losses.

Partition column chromatography of NF 1: This fraction remained pigmented and too oily for paper chromatography. A column of 15 grams of Hyflo Super-Cel was prepared,

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3. Allopregnane-11β,21-diol-3,20-dione-21-acetate,
4. 11β-hydroxy-Δ^4-androstene-3,17-dione.
5. Adrenosterone,
6. 11β-hydroxy-testosterone-acetate

R.O. is the runoff collected during 4 hours. It was re-applied in the Ligroin: Propylene glycol system (Ligr.:P.G.).

RRVS, RRS, RRM, and RRF are re-runs in the Bush B4 system of the very slow, slow, medium, and fast moving areas respectively, taken from the benzene-formamide chromatogram as shown.

with 75% methanol as stationary phase, and toluene+10% ethyl acetate as mobile phase. This system had been found to effect a rapid separation of less polar from more polar constituents, but the latter are eluted only after long development.

The first 50 ml. of eluate was highly pigmented, and was collected separately and labeled NF 1, Less Polar. The next 225 ml. of eluate, judged from other experiments to contain all more polar steroids, was labeled NF 1, More Polar.

Hot acid hydrolysis (NF 2): To the extracted urine at pH 0.8 was added 5% by volume of concentrated sulfuric acid. The mixture was heated in an open bottle in a boiling water bath in the hood 20 minutes after attaining 90° C, in order to split any remaining steroid conjugates. After 24 hours of continuous ether extraction, the neutral fraction was prepared as previously described, giving NF 2.

Paper chromatography: The benzene:formamide system of Burton *et al.* (13) was used for NF 1, More Polar. These chromatograms are schematically deplicted in Figure 2. The runoff of the "fast" benzene-formamide paper, as well as NF 1, Less Polar, were run in ligroin:propylene glycol (14). The NF 2 fraction, obtained after hot acid hydrolysis, was assumed to contain only less polar steroids, and was also run in ligroin:propylene glycol.

Spot tests and re-chromatography: Each chromatogram was examined under short wave ultra-violet light; (non-specific fluorescence usually made this test unreliable). The benzene-formamide papergrams of more polar steroids were also tested for reducing steroids with TPT. Six sections were eluted and 5 of them rechromatographed on narrower papers in the Bush B 4 system. (See Fig. 2.) Three strips 0.15 cm. wide were cut from each of these papers, and tested in turn by the soda-fluorescence reaction, reduction of TPT, and fluorescence under long wave ultra violet light when treated with concentrated sulfuric acid.

Chromatograms of less polar fractions, depicted in Figure 3, were tested only with alkaline m-dinitrobenzene (Zimmermann reagent) (14). Thus, only preformed 3- or 17-ketones were detected. Reactive areas were eluted and rechromatographed in either

TABLE 2. QUANTITATIVE DETERMINATION AND ESTIMATES OF STEROIDS IN CHROMATOGRAPHIC FRACTIONS OF EXTRACTS OF MOUSE URINE*

Type of steroid	Mobility	How assayed	Mouse urine content	
			Total	per 24 hrs.
			μg.	μg.
Highly polar Corcosteroids	Slower than Tetrahydro F	Quantitative Silber-Porter	30.0	0.20
Polar corticosteroids	Near cortisol	Quantitative Silber-Porter	10.4	0.07
Less Polar Corticosteroids	Near cortisone	Estimated from spot tests	25.0	0.18
Least polar Corticosteroids	Near corticosterone	Estimated from spot tests	53.0	0.35
Total corticosteroids			118.4	0.80
$C_{19}O_2$, 3- or 17-ketosteroids	Near 11-ketoetiocholanolone	Quantitative Micro-Zimmermann Reaction	3,360.	24.2
$C_{19}O_2$, 3- or 17-ketosteroids	Near or faster than dehydroepiandrosterone	Quantitative Micro-Zimmermann Reaction	1,542.	10.6
Total C_{19} 3- or 17-Ketosteroids			5,270.	34.6

* All urine came from female mice bearing ACTH-producing tumors. The total collection represented 150 24-hour samples.

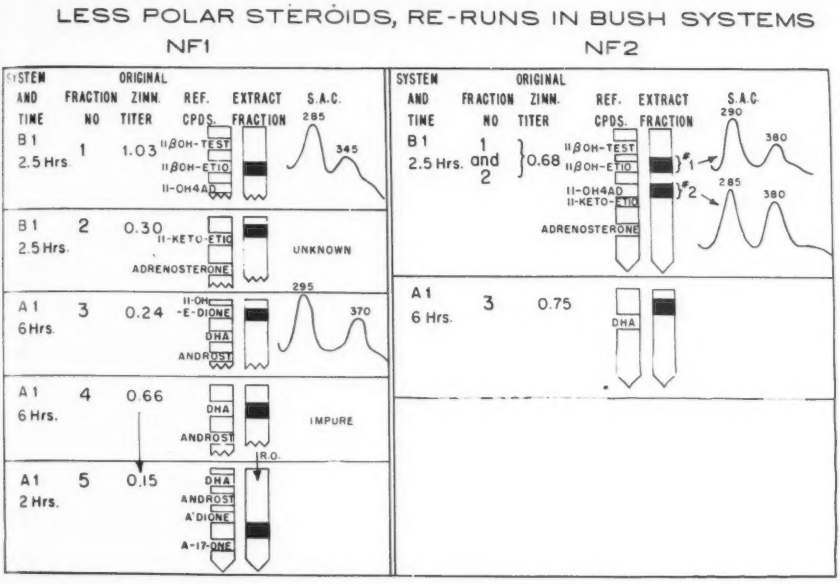

FIG. 3. Re-chromatograms of the less polar steroids of mouse urine in Bush systems, after elution from ligroin-propylene glycol chromatograms.

NF 1 is the neutral fraction of the extract made after hydrolysis with β-glucuronidase and continuous ether extraction at pH 0.8.

NF 2 is the neutral fraction of the extract made after hot acid hydrolysis.

Extract papers were tested with alkaline m-dinitrobenzene only, indicating either 3- or 17-ketosteroids.

S.A.C. is absorption spectrum of the sulfuric acid chromogen.

The "original Zimmermann titers" were obtained by quantitative micro colorimetric assays of eluates of regions on the previous ligroin:propylene glycol papergrams which gave the Zimmermann spot test.

Abbreviated names of reference standards are: TEST is testosterone, ETIO is etiocholanolone, E-DIONE is etiocholanedione, ANDROST is androsterone, A'DIONE is androstanedione. A-17-ONE is androstane-17-one. Other abbreviations are given in footnote 3.

the Bush B 1 (ligroin:toluene:methanol:water, 50:50:70:30), or "A 1" (ligroin:methanol:water, 100:90:10) systems.

Quantitative results of urine analysis:

Quantitative estimates of the various types of steroids found are given in Table 2. The total corticosteroid excretion, as deduced from Silber-Porter assays on eluates and reduction tests on the papergrams, was about 0.8μg. per mouse per day. The major corticosteroid component moved near corticosterone. Lesser amounts of more polar corticosteroids were also seen.

By far the dominant type of steroid was of C_{19} structure. Almost 35 μg. per day were excreted, or more than 40 times the corticosteroid total.

TABLE 3. SUMMARY OF THE CHIEF STEROIDS FOUND IN THE URINE OF MICE WITH AN ACTH PRODUCING TUMOR

Fraction	Structure assigned	Identification
NF 1-Corticosteroids*		
RRF #2	21-deoxy-20-hydroxy-pregnane, with Δ^4-3-ketone group	Tentative
NF 1-C_{19} Steroids		
Fraction #1	Unknown $C_{19}O_3$	—
#2	Unknown $C_{19}O_2$	—
#3	11β-OH-androstane-3,17-dione‡	Probable
#4	Unknown $C_{19}O_3$ or $C_{19}O_2$	—
#5	Unknown $C_{19}O_2$ or $C_{19}O$	—
NF 2†-C_{19} Steroids		
Fraction #1	11β,17α-dihydroxy-Δ^4-androstene-3-one	Tentative
#2	11β-hydroxy-Δ^4-androstene-3,17-dione	Probable
#3	Unknown $C_{19}O_3$ or $C_{19}O_2$	—
#4	Unknown $C_{19}O_2$ or $C_{19}O$	—

* NF 1—Neutral fraction obtained after hydrolysis with β-glucuronidase, acidification to pH 0.8, and continuous ether extraction.

† NF 2—Neutral fraction obtained after hot acid treatment of the residual urine from NF 1.

‡ Or 11β-hydroxy-etiocholane-3,17-dione.

About 70% of these were $C_{19}O_3$ steroids, as judged from chromatographic mobilities.

Qualitative results of urine analysis:

Several of the major bands found by spot tests were characterized by further procedures. Corticosteroids were sought only in NF 1; Zimmermann reacting, C_{19} steroids in both NF 1 and NF 2. The conclusions drawn from these procedures are summarized in Table 3.

More polar corticosteroids: (See Figure 2.) The positive Silber-Porter reaction given by eluates of the re-runs of the very slow (RRVS) and slow (RRS) regions, together with positive soda-fluorescence tests, indicate the presence of Δ^4-3-ketones and 17,21-dihydroxy-20-ketones, with additional oxygen groups.

Less polar corticosteroids: (See Figure 2.) The re-run of the original "medium" region (RRM #1) gave an area fluorescing in sulfuric acid opposite cortisol. When tested for aldosterone by bioassay,[4] this eluate was negative at the 1 μg. level. RRM #2 had a mobility and reactivity suggesting tetrahydrocorticosterone, which moves near cortisone.

Least polar corticosteroids: The most abundant corticosteroid material was found just below the corticosterone standard (RRF #2). It was characterized by an intense yellow fluorescence in the sulfuric acid spot test, and a positive soda-fluorescence reaction for a Δ^4-3-ketone. The presence of corticosterone was disproved by acetylation, inasmuch as the acetate moved much faster than corticosterone acetate (Figure 2). Its sulfuric acid absorption spectrum (S.A.C.) had a sharp peak at 295, and inflections at 345 and 420 mμ. The acetate was hydrolyzed with a rabbit liver enzyme

[4] We are greatly indebted to Dr. Grant Liddle who performed the bioassay for aldosterone.

preparation, in parallel with 2 μg. of corticosterone acetate. Whereas the latter was readily detected thereafter by a micro formaldehydogenic assay (15), the unknown yielded no formaldehyde. The presence of a polar C_{19} steroid was made unlikely by a negative CrO_3-KS reaction (11). An unknown 17,21-deoxy,20-hydroxy-pregnane compound, probably with a Δ^4-3-ketone group is therefore possible. No such substance was available for comparison, but several similar 21-deoxy steroids reacted in the sulfuric acid-fluorescence spot test, making this structure a possibility. The weak TPT reduction test is not accounted for; it may have been due to another constituent.

C_{19} steroids, NF 1:

Only areas reacting in the alkaline m-dinitrobenzene spot test were followed. The reacting bands on the ligroin-propylene glycol papers were cut out and quantitatively assayed by the micro Zimmermann reaction (16). Figure 3 shows the individual titers on those fractions which were rechromatographed and further characterized.

NF 1, Fraction 1: (Fig. 3.) A band which had moved only 8 cm. after 96 hours in ligroin-propylene glycol, assayed 1.03 mg. It ran directly opposite 11β-hydroxy-etiocholanolone in the Bush B 1 system. It gave a brilliant instant bluish reaction in the Zimmermann test, which quickly turned to a blackish green. The S.A.C. had maxima at 285 and 345 mμ, indicating a highly polar unknown C_{19} steroid, with a Δ^4-3-ketone group. This was the most plentiful component of all those found in the mouse urine. It was not further characterized.

NF 1, Fraction 2: Assaying 0.3 mg., this material ran opposite 11-ketoetiocholanolone in both systems, but its S.A.C. indicated an unknown compound.

NF 1, Fraction 3: Assaying 0.24 mg., this band had mobilities in both systems and an S.A.C. curve entirely consistent with the presence of 11β-hydroxy-androstane (or etiocholane)-3,17-dione.

NF 1, Fraction 4: This fraction, assaying 0.66 mg., had run off the 24 hour ligroin-propylene glycol paper, as had androsterone and dehydroepiandrosterone (DHA). On rechromatography in the Bush A 1 system, its mobility was the same as that of DHA, but was unchanged by acetylation. Hence DHA could not have been a major component. The S.A.C. was unlike that of DHA and could not be further interpreted. Since it moved faster than either etiocholanetrione or androstanetrione, these were eliminiated as possibilities. An unknown fast $C_{19}O_3$ or slow $C_{19}O_2$ steroid is indicated.

NF 1, Fraction 5: The runoff from the 6 hour A 1 chromatogram of Fraction 4 assayed 0.15 mg. When re-run in the same system for 2 hours, it yielded a strong band just below androstanedione and above androstane-17-one. These properties are consistent with the presence of an unsaturated $C_{19}O$ steroid such as $\Delta^{3,5}$-androstadiene-17-one, or Δ^2-androstene-17-one.

C_{19} steroids, NF 2: (After hot acid hydrolysis, Fig. 3.)

NF 2, Fraction 1 and 2: An area moving near 11-OH4AD in ligroin-propylene glycol gave a diffuse Zimmermann reaction and assayed 0.68 mg. On rechromatography in the Bush B 1 system, 2 reactive bands were found, #1 slightly below 11β-hydroxy-testosterone and opposite 11β-hydroxy-etiocholanolone, and #2 opposite 11-OH4AD.

NF 2, Fraction #1 gave a weak, greyish blue Zimmermann spot test. On acetylation and rechromatography it ran faster than acetylated 11β-hydroxytestosterone. (Rf = .93 vs. .70, ratio = 1.32.) An S.A.C. of the acetate had good peaks at 290 and 380 mμ con-

sistent with the presence of 11β-hydroxytestosterone (17). After oxidation of the acetate with chromic acid (6), a single sharp S.A.C. peak at 290 mμ was obtained. By analogy with epitestosterone (whose mobility is faster than that of testosterone, ratio = 1.35 (18)), these properties suggest the presence of 11β, 17α-dihydroxy-Δ^4-androstene-3-one.

NF 2, Fraction #2: The free compound gave an S.A.C. with excellent peaks at 285 and 380 mμ, essentially identical with that of 11-OH4AD. (See Figure 3.) The free compound was treated with acetic anhydride and pyridine in parallel with several steroids with acylable hydroxyl groups, and 11-OH4AD, which has no acylable hydroxyl groups. On rechromatography the former compounds were found to be completely acetylated, whereas NF 2 Fraction 2 had the same mobility as both free and acetylated 11-OH4AD. This evidence is considered a highly probable identification of 11-OH4AD, (11β-hydroxy-Δ^4-androstene-3,17-dione).

DISCUSSION

These studies represent the first detailed analysis of steroids in the blood and urine of mice. Previous analyses of adrenal vein blood and plasma from rats (12, 19) have shown that cortisol is absent or present only in traces. A substance whose mobility, reactions in spot tests, and fluorescence indicated it to be corticosterone was the dominant steroid. Our analysis of mouse serum gave results similar to those reported for rats, though it cannot be positively stated that the fluorescing component was corticosterone on the basis of mobility alone. The findings of Hoffman (20) on *in vitro* adrenal steroid synthesis in the albino mouse are almost identical with our results on normal mouse serum. Paralleling the observation of Bush (12) that 11-OH4AD constituted up to 40% of the total Δ^4-3-ketonic steroids in rat adrenal vein plasma, our experiments point to 11-oxygenated C_{19} steroids as the most abundant components of mouse serum.

The effect of transplanted ACTH producing tumors on the composition of mouse serum could be plainly discerned. Both the "cortisol-like" and "corticosterone-like" components were doubled in amount, as were the less polar steroids reacting in the CrO_3-KS test. However, the more polar CrO_3-KS reactants were not discernibly increased. It is noteworthy that even the prolonged stimulation with ACTH had not appreciably altered the composition of the adrenal cortical secretion in favor of cortisol, as has been reported for rabbits receiving exogenous ACTH (21).

Urine collected from 3 tumor bearing mice provided a richer source of steroids from which to draw conclusions as to the adrenal cortical secretion. Possible effects of storage at pH 8.9 cannot be assessed, but must be kept in mind. Chromatographic fractions of more polar corticosteroids giving typical colors with phenylhydrazine (Silber-Porter) suggest that the mouse adrenal is capable of 17-hydroxylation, as well as 11- and 21-hydroxylation, though to a far lesser extent. Corticosterone itself was not found in the urine, but substances which could be metabolites of corticosterone were the major corticosteroids. Band "RRM #2" could have contained tetrahydrocorticosterone, whereas "RRF #2" appeared to consist of a corti-

corticosterone derivative which had been reduced at both C-20 and 21 to form a 21-deoxy-20-hydroxy steroid. A substance with a similar side chain, 3α, 20α-dihydroxy-pregnane-11-one has been isolated from the urine of a human subject treated with corticosterone (22). No evidence for the presence of Reichstein's substance S or DOC was obtained.

The finding that $C_{19}O_3$ steroids are by far the dominant components in mouse urine gives support to the evidence for their presence as major constituents of blood serum. It seems likely that they are derived from a primary adrenal cortical precursor such as 11-OH4AD since there is as yet no evidence that 17-desoxycorticosteroids such as corticosterone are metabolized in this way. The possibility of their being metabolites of an 11,17-oxygenated precursor such as cortisol seems unlikely as the blood serum contained so little such material.

The identification of 11-OH4AD in the urine of the tumor bearing mice thus suggests that either this steroid or 11β-hydroxy-testosterone may be a primary adrenal secretory product. Other $C_{19}O_3$ steroids which appeared to be 11β-hydroxy-androstane-3,17-dione and 11β,17α-dihydroxy-Δ^4-androstene-3-one could then be metabolites of these compounds. However, it must be recalled that the major urinary $C_{19}O_3$-like steroid was an unknown substance more polar than 11-OH4AD. Dehydroepiandrosterone was not found (though two unknown ketosteroids of similar mobility were present), nor was there any evidence for the excretion of androsterone or etiocholanolone.

The question of what alterations in adrenocortical secretion were responsible for the hyperadrenocorticism and other symptoms of these mice remains difficult to answer. The steroid metabolites found in urine were quite different from those of humans having similar symptoms. The mice gave signs of possible excessive cortisol secretion (thymic atrophy, lymphopenia and obesity), but no large amounts of cortisol metabolites were found. It is possible nevertheless that the relatively small amounts of cortisol-like and corticosterone-like materials found in both blood and urine were responsible for the "glucocorticoid" effects. The electrolyte effects (polydipsia, polyuria) may have resulted from an elevated secretion of corticosterone (23, 24) since no aldosterone was found.

Alterations in the female reproductive tract (cessation of estrus cycling, ovarian atrophy, and partial mucification of the vagina) may have been caused by unknown precursors of the C_{19} metabolites. These were probably weak or inactive as androgens, since the seminal vesicles and ventral prostate were not maintained in hypophysectomized or castrated tumor bearing mice (1). Yet the vaginal and ovarian changes suggest inhibition of pituitary gonadotrophins. Dorfman and Shipley (25) give data indicating that C_{19} steroids with weak androgenic activity (as measured by prostate and capon comb effects) are capable of inhibiting the rat pituitary. Thus

the cessation of estrus cycling seen in our mice may have been the indirect result of the excessive production of C_{19} steroids. That the partial vaginal mucification was a direct effect was shown when complete mucification occurred in hypophysectomized tumor bearing mice. Huggins *et al.* (26) in studying the effects of a large group of C_{19} steroids found that 11-OH4AD is among those capable of producing mucification. Since this compound or a closely related one was most probably secreted by the adrenal glands of the tumor mice, the mucification can be explained on this basis. Although it has been suggested that dehydroepiandrosterone is an active androgen in mice (27) it was not detected in the present study.

It is of interest that two of the most plentiful steroids found only after hot acid hydrolysis were Δ^4-3-ketones (11-OH4AD and probably 11β,17α-dihydroxy-Δ^4-androstene-3-one). In human urine Δ^4-3-ketones are usually found in the non-conjugated moiety and only in relatively small amounts. They seldom appear in an extract made after heating with strong acid. Salamon and Dobriner (28) have reported the isolation of 11-OH4AD from the urine of a human subject treated with ACTH. It was obtained by continuous ether extraction of urine acidified to pH 1.0. Since our fraction NF 1 was similarly extracted but the urine did not yield 11-OH4AD except after further acidification and heating (NF 2), an unknown form of conjugation in mice is suggested. In fact, both known forms of conjugates, glucuronides and sulfates, should have been hydrolyzed before hot acid hydrolysis by our procedures, so that all additional steroids obtained in NF 2 reflect unknown conjugates.

The finding of appreciable amounts of steroids in the urine of mice is in accord with previous work (2, 4, 5). Karnofsky *et al.* (29) also have reported excretion levels of 17-ketosteroids based on assays of crude extracts. This is in contrast to the situation in rats, where the urinary excretion is very small (30, 31) and does not rise on administration of sufficient ACTH to lower the eosinophile account.[5] It can only be surmised what proportion of either the total adrenocortical secretion or total urinary excretion of the mice is represented by the recovered steroids in our study.

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[5] In unpublished experiments (H. Wilson) it was found that extraction of "14 days" of rat urine with chloroform after incubation with β-glucuronidase gave extremely low titers and atypical colors in the usual colorimetric assays. Administration of ACTH did not affect the corticosteroid titers. Acid hydrolyzed 17-ketosteroid titers were very slightly raised by ACTH and very slightly depressed after bilateral adrenalectomy.

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THE KETOGENIC ACTIVITY OF CORTICOTROPIN, A PRESUMED EXTRA-ADRENAL ACTION[1]

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ABSTRACT

The ketogenic response to protein ACTH, oxycel-adsorbed ACTH, Corticotropin A, mixtures of purified corticotropin peptides, pepsin and chymotrypsin digestion products of β-corticotropin, β-MSH and commercial vasopressin has been tested in intact rats, according to techniques previously described. It has already been shown that corticotropin induces ketosis in adrenalectomized rats and hence the response studied is presumed to be an extra-adrenal one. Oxycel corticotropin, Corticotropin A and various mixtures of corticotropin peptides ($\alpha_1-\alpha_2$ and γ-pool corticotropin, American Cyanamid Company) exhibited high ketogenic activity whereas a protein ACTH (Armour), three products of pepsin digestion and one of chymotrypsin digestion and fraction δ-1 of the American Cyanamid Company had markedly decreased activity. The ketogenic active corticotropins also were known to have ascorbic acid depleting and MSH activities, as did the peptides from pepsin digestion. The chymotrypsin peptide was devoid of biological activity. Treatment of ketogenic active peptides by boiling in 0.1N NaOH resulted in loss of ascorbic acid depleting and ketogenic activities but not MSH activity whereas mild alkali treatment had no effect on these activities. β-MSH had no ketogenic or ACTH activity. The similarity in structure between alkali-boiled ACTH and of β-MSH is noted. Ketogenic activity of ACTH peptides may be reversibly inactivated by H_2O_2 and cysteine, as has been reported for the adrenal ascorbic acid depleting and MSH activities of ACTH. Vasopressin had no ketogenic activity. The data are interpreted as indicating that ketogenic activity is an intrinsic property of the ACTH peptide molecule. The significance of these results in relation to the peptide structures of ACTH and MSH is discussed.

INVESTIGATIONS from two laboratories have established that certain preparations of corticotropin have ketogenic and adipokinetic activities when tested in rats or mice (1, 2, 3, 4). These metabolic responses have been demonstrated to occur in adrenalectomized animals and hence they must be interpreted as extraadrenal responses to the corticotropin preparations. This interpretation is supported by the observations that cortisol and cortisone suppress fasting and other types of ketosis, while adrenal insufficiency accentuates ketosis during fasting (5, 6). Data have been presented already

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which make it unlikely that the ketogenic and adipokinetic responses to corticotropin are due to contamination with either growth hormone or thyrotropin or some independent factor capable of inducing these metabolic responses (3). In an earlier report on the ketogenic response to corticotropin we interpreted this response as probably not being a property of the corticotropin molecule per se (2). In the present study observations were made with more highly purified samples of corticotropin, including corticotropin A, various pepsin and chymotrypsin digestion products of β-corticotropin and other by-products in the fractionation of oxycel corticotropin. The results strongly suggest that the ketogenic action is indeed a response to the corticotropin molecule itself, an action which presumably is extra-adrenal although the present studies were done in intact animals.

METHODS AND MATERIALS

Ketogenic activity was assayed in 24-hour fasted male rats under light nembutal anesthesia by estimating the increase in blood ketone levels 3½ hours after intraperitoneal injection of the test material, as described in detail in the previous reports (2, 3). In a few instances, observations were made in unanesthetized rats.

The following corticotropin preparations were used:

1. Armour Laboratories:
   a. ACTHAR-A—Lot J-17409. Protein ACTH, 1 mg. equivalent to 1 unit LA-1-A.
   b. Porcine corticotropin A—Lot 980-001-1 (7)
2. Lederle Laboratories, American Cyanamid Co. (8, 9, 10)
   a. Oxycellulose adsorbed ACTH—Lot 10-OC 12.5 LAC 5413-159; 80-100 U.S.P. units/mg.
   b. α_1-α_2 pool—Lot S1079-28; 80-100 U.S.P. units/mg.
   c. γPool—Lot S1079-45; 80-100 U.S.P. units/mg.
   d. δ_1—Lot S1079-24; 10 U.S.P. units/mg.
   e. $\beta P_s(3)$—Lot S-1892-34 No. 225—Pepsin digest of β-ACTH; 80-100 U.S.P. units/mg.
   f. $\beta P_s(3)$—Lot 1892-34—Pepsin digest of β-ACTH; 80-100 U.S.P. units/mg.
   g. $\beta P_s(4)$—Lot S776-24—Pepsin digest of β-ACTH; 80-100 U.S.P. units/mg.
   h. C-10-Chymotrypsin digest of β-ACTH—no ACTH activity.

The corticotropins were dissolved in distilled water just prior to use. Inactivation studies were carried out as follows:

1. NaOH—The hormone (0.5–1.0 mg.) was dissolved in 1.0 ml. of 0.1N NaOH and either (a) allowed to stand at 25° C for 24 hours or (b) heated to 100° C for 20 minutes. The solution was then brought to pH 5.0 with 0.1N HCl and diluted with distilled water so that the chosen dose was contained in a volume of 0.1–0.3 ml.
2. Reversible inactivation with H_2O_2 and cysteine (11, 12, 13). 1 mg. of the α_1-α_2 pool was dissolved in 1 ml. of 0.2 M. phosphate buffer, pH 6.7. 0.05 ml. of H_2O_2 (Superoxol) was added and allowed to stand for 30 minutes. At the end of this time 0.05 ml. of a solution of catalase (Worthington) containing 1500 μ/ml. was added and the solution shaken until bubbling stopped. Addition of 0.01 ml. of catalase solution caused no further release of gas. 0.575 ml. of this solution was diluted to 5.0 ml. and tested for ketogenic activity. For reactivation 0.575 ml. of 0.2 M. cysteine HCl was added to 0.575 ml. of the H_2O_2 treated hormone solution, the pH adjusted to 4.1 and the solution incubated at 72° C for 19 hours. The solution was then brought to pH 6.8 and diluted to 5.5 ml. for injection.

RESULTS

Table 1 records the ketogenic response to protein and oxycel ACTH compared to corticotropin A, the α_1-α_2 and γ pools, δ-1, and the 3 pepsin and the one chymotrypsin digestion products of β-corticotropin. The data on the oxycel corticotropin are taken from a previous paper (3).

The protein ACTH was devoid of ketogenic activity at a dose of 10 mg. (10 units). By contrast, the oxycel corticotropin was active at 0.020 mg.,

TABLE 1. INFLUENCE OF CORTICOTROPINS ON RISE IN BLOOD KETONES IN 3½ HOURS

Corticotropin preparations	No. of rats	Dose in mg.	Mg.% increase as acetone	P
Control	21	—	+ 3.6±0.31	—
Armour Protein ACTH—J17409	8	10.0	+ 3.1±0.57	n.s.
Oxycel	6	0.030	+ 8.5±1.27	<.01
Oxycel	6	0.010	+ 3.8±0.79	n.s.
α_1-α_2 Pool	11	0.030	+11.2±1.95	<.01
α_1-α_2 Pool NaOH 24′ 25°*	7	0.030	+10.9±1.24	<.01
α_1-α_2 Pool NaOH 20″ 100°	8	0.030	+ 1.2±0.43	<.01
α_1-α_2 Pool	11	0.010	+ 6.7±0.91	<.01
α_1-α_2 Pool NaOH 24′25°	10	0.010	+ 5.4±0.62	<.01
α_1-α_2 Pool	6	0.003	+ 3.6±0.73	n.s.
Corticotropin-A	8	0.020	+ 8.3±1.25	<.01
γ-Pool	11	0.030	+10.2±1.81	<.01
γ-Pool NaOH 24′ 25°	7	0.030	+10.4±0.75	<.01
γ-Pool	10	0.010	+ 9.2±0.87	<.01
γ-Pool	10	0.005	+ 4.1±0.59	n.s.
γ-Pool NaOH 24′25°	8	0.005	+ 4.4±0.84	n.s.
$\beta P_s(3)$ Lot S-1892-34-225	6	0.200	+ 3.2±0.50	n.s.
$\beta P_s(3)$ Lot S-1892-34-225	8	0.100	+ 4.1±0.86	n.s.
$\beta P_s(3)$ Lot S-1892-34-225	8	0.060	+ 5.3±0.85	n.s.
$\beta P_s(3)$ Lot S-1892-34-225	14	0.030	+ 5.7±0.58	<.01
$\beta P_s(3)$ Lot S-1892-34-225	9	0.010	+ 4.8±0.89	n.s.
$\beta P_s(3)$ Lot S-1892-34	10	0.200	+ 6.5±0.82	<.01
$\beta P_s(3)$ Lot S-1892-34	5	0.100	+ 2.9±0.31	n.s.
$\beta P_s(4)$ Lot S-776-147	5	0.200	+ 5.9±0.25	<.02
δ-1	5	0.200	+ 4.5±0.51	n.s.
δ-1	9	0.030	+ 4.5±0.80	n.s.
C-10	13	0.200	+ 5.3±0.64	<.05

* See text for description of NaOH inactivation.

but not at 0.010 mg., corticotropin A at 0.020 mg. (because of limitations of supply, only one dose was tested), and the α_1-α_2 and γ pools at 0.010 mg. Of the three pepsin digests of β-corticotropin, all showed a substantial decrease in activity compared to the above preparation. $\beta P_s(3)$—Lot S1892-34-225 was inactive up to 0.200 mg. although there was a small, but statistically significant response to the 0.030 mg. dose. This remains unexplained, but is probably of no biological significance in view of the repeatedly negative responses to larger doses. $\beta P_s(3)$—Lot S1892-34 was active at 0.200 mg. but not at 0.100 mg. $\beta P_s(4)$—Lot S776-147 showed some activity at 0.200 mg. but this was not striking. Other doses of these pepsin digests could not be tested since their supply was exhausted. The chymotrypsin digest of β-corticotropin, C-10, had dubious activity ($P < .05$) at a dose of 0.200 mg. The δ-1 fraction was devoid of activity.

We have already reported that corticotropin is much more active in anesthetized than in unanesthetized rats (2, 3). For this reason a few observations in unanesthetized rats were made with some of the above preparations. The α_1-α_2 pool was ineffectual in doses less than 0.200 mg. The latter dose induced a small but significant response while 0.400 mg. yielded an unequivocal increase in ketonemia. This compares to the significant ketonemic response to 0.010 mg. in the anesthetized rat. A single dose of the γ pool (0.100 mg.) was tested and found ineffectual. These data thus confirm previous results with various samples of oxycel corticotropin.

In order to gain further insight into the relation between ketogenic and corticotropin activity, observations were made on the effect of various inactivation procedures on these biological activities. We have previously found that the ketogenic activity of oxycel corticotropin always resisted exposure to 0.1N NaOH for 16–24 hours at 25° C but this activity was destroyed by more vigorous alkali treatment (3). As seen in Table 1, the α_1-α_2 pool and the γ pool likewise resisted the mild treatment, whereas on exposure of the α_1-α_2 pool to 100° C for 20 minutes, ketogenic activity was not only completely lost but the boiled peptide actually inhibited ketosis. It might be noted too in passing without presenting the data that the alkali boiled α_1-α_2 pool was likewise inactive when tested at a dose of 0.400 mg. in unanesthetized rats. Dr. George Sayers, Cleveland, kindly carried out an adrenal ascorbic acid depletion assay of the α_1-α_2 pool treated just as above. Exposure to 0.1N NaOH for 24 hours at 25° C resulted in only a small loss, whereas after 20 minutes at 100° C less than 1% of ACTH activity remained.

As a further indication of the close association between ACTH and ketogenic activities, the technique of reversible inactivation with H_2O_2 and cysteine was employed (11, 12, 13). Table 2 shows that treatment of the α_1-α_2 pool with H_2O_2 resulted in complete loss of ketogenic activity. When the inactivated hormone was incubated with cysteine, ketogenic activity was restored. With the smaller dose (0.030 mg.) the cysteine treated material was considerably less active than the starting material, yet was significantly more active than the control. The failure to restore full activity is in keeping with the reported order of reactivation of ACTH as

TABLE 2. REVERSIBLE INACTIVATION OF α_1-α_2 ACTH WITH H_2O_2 AND CYSTEINE*

Treatment	No. of rats	Dose in mg.	Mg.% increase as acetone	P
Control	12	—	+ 3.5±0.29	—
α_1-α_2 Pool	11	0.030	+11.2±1.95	<.01
α_1-α_2 Pool H_2O_2	8	0.030	+ 4.4±0.54	n.s.
α_1-α_2 Pool H_2O_2	7	0.100	+ 3.3±0.68	n.s.
α_1-α_2 Pool H_2O_2 cysteine	16	0.030	+ 6.1±0.68	<.01
α_1-α_2 Pool H_2O_2 cysteine	6	0.100	+13.3±2.80	<.01

* See text for description of inactivation.

measured by the adrenal ascorbic acid depletion assay (13). The latter was not carried out in the present experiment, but the correspondence of the results on ketogenesis with those on ACTH activity recorded in the literature is sufficiently close to leave little doubt concerning its significance.

DISCUSSION

The interpretation of these data is contingent on the clarification of the chemical nature of the corticotropin samples investigated. Figure 1 presents a flow diagram illustrating the derivation of the various samples from the fractionation of porcine oxycel corticotropin by Bell, Shepherd and their colleagues at the Lederle Laboratories (8, 9, 10). Included in the diagram are notations of corticotropic and melanophore stimulating activities as estimated at the Lederle Laboratories and ketogenic activities as recorded in this report. The original oxycel ACTH had all these activities, as indicated. By countercurrent distribution this material was separated into a rapidly moving fraction, T(6–10) and a slow moving fraction, B(1–5). The former comprised 80% of the starting weight and contained 90% of the ACTH activity and 10% of the MSH activity. The rapidly moving fraction was further subdivided into an α pool, β-corticotropin, which accounted for 49% of this fraction, a γ pool and a δ fraction. The α pool consists of a mixture of highly active peptides which presumably differ only in

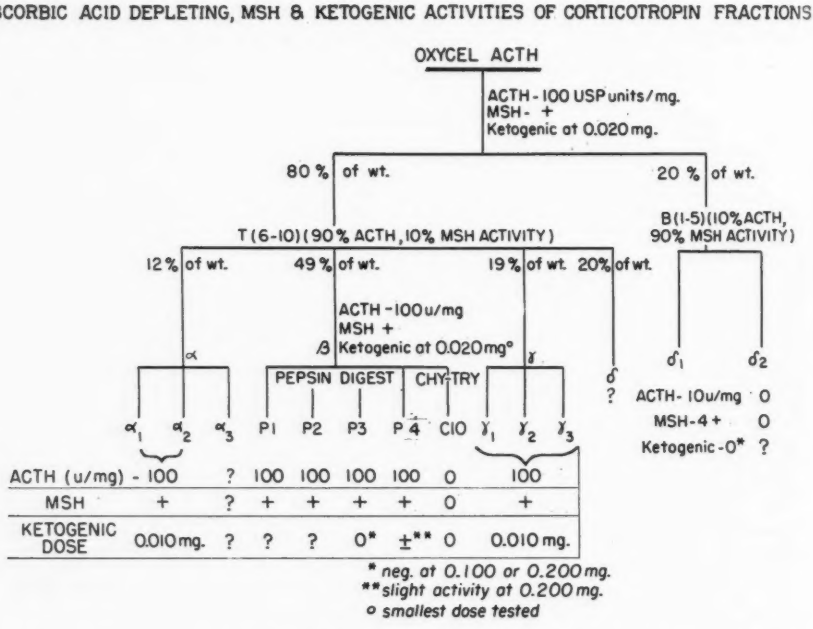
ASCORBIC ACID DEPLETING, MSH & KETOGENIC ACTIVITIES OF CORTICOTROPIN FRACTIONS
OXYCEL ACTH
ACTH - 100 USP units/mg.
MSH - +
Ketogenic at 0.020 mg.
80 % of wt.
20 % of wt.
T(6-10)(90% ACTH, 10% MSH ACTIVITY)
B(1-5)(10% ACTH, 90% MSH ACTIVITY)
12 % of wt.
49 % of wt.
19 % of wt.
20% of wt.
ACTH - 100 u/mg
MSH +
Ketogenic at 0.020 mg°
PEPSIN DIGEST
CHYMOTRY
ACTH - 10 u/mg
MSH - 4 +
Ketogenic - 0* ?
ACTH (u/mg) - 100 ? 100 100 100 100 0 100
MSH + ? + + + + 0 +
KETOGENIC DOSE 0.010 mg. ? ? ? 0* ±** 0 0.010 mg.
* neg. at 0.100 or 0.200 mg.
** slight activity at 0.200 mg.
° smallest dose tested

FIG. 1. Fractionation of Oxycel Corticotropin (8, 9, 10). See text for description.

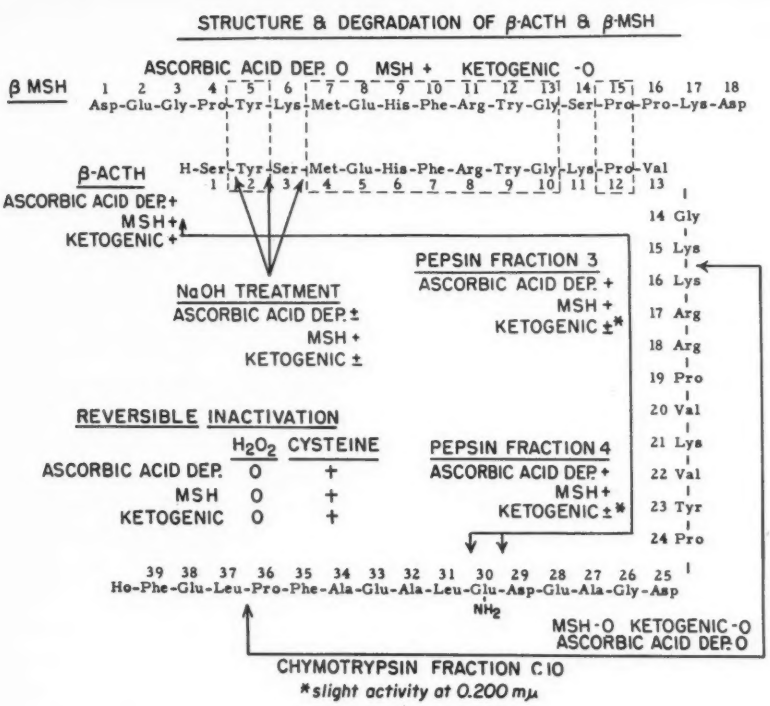

FIG. 2. Structures of β-Corticotropin (10) and β-MSH (14, 15) in relation to biological activities.

minor ways from β-corticotropin, which has been completely characterized (10) (Fig. 2). The α_1-α_2 pool had all three activities. β-Corticotropin from the Lederle Laboratories was not available for testing but a sample of Armour corticotropin-A, which was presumably identical with Lederle's β-corticotropin (7), exhibited high ketogenic activity. The β-corticotropin was subjected to graded pepsin and chymotrypsin digestion. The pepsin digest products (P3 and P4) retained their original ACTH and MSH activities but showed a marked loss of ketogenic activity. The chymotrypsin fraction (C-10) was devoid of biological activity.

The γ pool behaved similarly to the α pool with respect to the three biological activities. No information is available about δ, but δ-1, which had much reduced ACTH activity (10 units/mg.), was essentially devoid of ketogenic activity and had considerably enhanced MSH activity.

Additional information concerning the significance of these observations may be derived from an examination of the structure of β-corticotropin and consideration of the influence of various chemical procedures on it and on its biological activities. Figure 2 diagrams the structure of β-corticotropin which has been determined to be a peptide comprising 39 amino acids (10)

and compares it with that of β-MSH recently reported by Harris and Roos (14) and confirmed by Geschwind *et al.* (15). These investigators have already pointed out that the correspondence between certain amino acid sequences in the two hormones may account for the intrinsic MSH activity of the corticotropin peptides. The studies of Shepherd *et al.* (8) have demonstrated that the N-terminal end of β-corticotropin is essential for its adrenal ascorbic acid depletion activity. Boiling in 0.1N NaOH progressively removes the first three amino acids (8) and results in loss of ACTH and ketogenic activities, but leaves MSH activity, as might be anticipated from the structures of the β-ACTH and β-MSH molecules. The milder alkali treatment apparently does not result in a significant change in the molecule and there is no loss of biological activity.

Both ACTH and MSH may be reversibly inactivated by oxidation with H_2O_2 and reduction with cysteine (11, 12, 13). The fact that the ketogenic activity of corticotropin behaves similarly suggests that the part of the molecule responsible for the latter activity may be common to ACTH and MSH. At the same time both adrenal ascorbic acid depleting and ketogenic activities seem to depend on the presence of the N-terminal serine, judging from the loss of both activities on boiling in 0.1N NaOH. It might be anticipated, then, that β-MSH would be devoid of ketogenic activity as it is of adrenal ascorbic acid depleting activity. Since the present work was completed we were fortunate to obtain a small amount of β-MSH from Drs. Geschwind and Li. At a dose of 0.100 mg. it was completely impotent in the ketosis stimulating test. Indeed, it actually inhibited ketosis (control $+4.33 \pm 0.56$, and β-MSH $+0.39 \pm 0.22$ mg.% in 3.5 hours), a finding of some interest in view of the similar inhibition by alkali inactivated α_1-α_2 corticotropin. Bell's fraction δ-1, which presumably is rich in β-MSH, was also inactive in ketosis.

The pepsin digestion products, P3 and P4, consisting of peptides comprising the first 30 and 29 amino acids of β-corticotropin respectively, retain full ACTH and MSH activity, but our data indicate marked loss of ketogenic activity. This finding must be interpreted cautiously since simultaneous ACTH assays were not done and hence we have no assurance that the fractions retained full adrenal ascorbic acid depleting activity at the time we tested them for ketogenic activity. Unfortunately, no more of these samples are available. Provisionally, the data suggest that more of the ACTH molecule is necessary for the ketogenic response than for adrenal ascorbic acid depletion since it seems unlikely that all three samples would have deteriorated. The chymotrypsin fraction represented a sequence toward the carboxyl end of the molecule and was largely inactive. This is not surprising in view of the previous data indicating the essentiality of the N-terminal serine for the ACTH and ketogenic actions.

In 1953 Ingle *et al.* (16) reported that some partially purified preparations of corticotropin exhibited an extra-adrenal action in the work performance test. Subsequently they found that corticotropin A and other

pure preparations of the hormone were devoid of this activity which was demonstrated to be due to contamination with vasopressin (17). We have tested Parke, Davis Vasopressin at a dose of 0.02 unit, the optimal dose reported by Ingle, and found it inactive in the ketosis assay. Since corticotropin A was highly active we consider it unlikely that we are dealing with the same factor which Ingle found to have an extra-adrenal action in the work performance test.

Taken in their entirety, these data give strong support to the interpretation that the ketogenic activity of ACTH is indeed an intrinsic property of this hormone, at least in its peptide form. This conclusion is in disagreement with an earlier interpretation that the two activities were probably separable (2). The latter conclusion was based on the discrepancy between the potencies of different ACTH preparations with respect to ketosis and ACTH units. In view of the uncertainties concerning the chemical nature of the hormones tested in the earlier work and the present evidence that protein ACTH is relatively impotent compared to peptide ACTH, it is evident that the earlier interpretation was based on an insecure foundation.

The finding that protein ACTH had no ketogenic activity even at a dose of 10 mg. (10 units) while other corticotropins were active in doses of 0.010–0.030 mg. (1–3 units) is of some interest. The lack of ketogenic activity was not associated with deterioration of adrenal ascorbic acid depleting activity since on reassay by the Armour Laboratory the original potency remained, although the sample had been in our laboratory for about 8 years. It is unlikely that slower absorption accounts for the lack of activity since growth hormone and thyroid stimulating hormone were active as proteins (3). The finding that an active protein ACTH does not exhibit a metabolic action possessed by more purified ACTH preparations gives some support to a suggestion previously made provisionally (4) that the extra-adrenal metabolic actions of ACTH might depend on the chemical constitution of the hormone molecule. It is conceivable that ACTH is secreted as a protein and fixed to the adrenal gland which in turn possesses a mechanism for converting the protein to a peptide which is metabolically active in the gland. When this same peptide is injected parenterally, it may not necessarily be as selectively fixed by the adrenal cortex and thus might be capable of initiating a variety of metabolic reactions in other tissues. Alternative interpretations of these data are possible and are discussed in detail elsewhere together with some of the implications of the multiple extra-adrenal metabolic actions of ACTH, which are at present under intensive investigation in this laboratory (4). In essence, we suggest that the extra-adrenal metabolic responses represent reactions to ACTH in other tissues which are analogous to those taking place within the adrenal cortex. It remains to be established whether these reactions occur in response to naturally secreted ACTH as well as to the extracted hormone which has been chemically purified and possibly modified in structure.

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Note Added in Proof

Since submitting this manuscript we have learned of two reports that pepsin digests of corticotropin retain adipokinetic activity, although our data point to substantial loss of ketogenic activity. Li *et al.* (J. Exp. Med. 105: 335, 1957) report that pepsin digests of α-corticotropin have adipokinetic activity while chymotrypsin digests do not. Dr. S. Steelman (personal communication) informs us that Bell's pepsin fractions P-2, P-3 and P-4 all possess adipokinetic activity in the mouse and this activity is approximately equivalent to that of the α, β, and γ fractions. Unfortunately, the lots used by Steelman were different from those employed in our study. Using a newly devised technique which measures release of non-esterified fatty acids from adipose tissue in vitro we have found that fraction P-3 (lot S-1892-34 No. 225) has lipolytic activity as do all the active ketogenic preparations tested. Fraction C-10 is inactive. (J. E. White and F. L. Engel, unpublished observations). The basis for the discrepancy between the adipokinetic-lipolytic and the ketogenic activities remains to be established.

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MECHANISM OF THE THYROID STIMULATION PRODUCED BY SODIUM CHLORIDE IN THE MOUSE[1]

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ABSTRACT

The mechanism by which prolonged ingestion of sodium chloride induces a stimulation of the thyroid in mice has been investigated.

Mice fed for 2 months on a low iodine diet containing 1% sodium chloride exhibited larger thyroid glands with a smaller iodine content than control mice fed the same diet without sodium chloride. Furthermore, radioiodine measurements performed at intervals revealed that the presence of sodium chloride in the diet increased the fraction of the body iodide excreted into the urine and decreased the fraction taken up by the thyroid.

In mice given radioiodine and receiving a single intragastric dose of 30 mg. sodium chloride, the amount of radioiodine excreted in the urine within the next 2–3 hours was augmented 10–20 times, while the radioiodine level in blood was lowered moderately. (Similar effects were observed in thyroidectomized but not in nephrectomized mice.) Twenty-four hours after sodium chloride administration, the radioiodine content of the thyroid decreased to one half the amount in controls.

These results indicated that sodium chloride increases the urinary loss of iodine and thus lowers the plasma level of this element. As a result, less iodine is available to the thyroid gland and less thyroid hormone is secreted. The pituitary gland responds by releasing more thyrotrophic homone and thus stimulates the thyroid gland.

ADDITION of sodium chloride to the diet of mice induces a stimulation of the thyroid gland characterized by hypertrophy, hyperplasia and hyperemia, with loss of colloid and increased iodine uptake (1). Furthermore, if the diet is low in iodine, sodium chloride enhances the severity of the resulting goiter (1). The present work was undertaken to elucidate the mechanism by which sodium chloride stimulates the thyroid gland.

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MATERIALS AND METHODS

The experiments were carried out with 2.0–6.5 month old C_3H/Jax male mice, fed on a basic low iodine diet containing 89% corn meal, 10% dried brewer's yeast, and 1% calcium carbonate (1). When needed, sodium chloride (reagent grade) was added so as to make up 1% of the diet, and the amount of corn meal was correspondingly reduced to 88%. Iodine was estimated in each batch of the foodstuffs by a modified Chaney procedure (2) following ashing of 5 gm. of food with 30 gm. of potassium carbonate at 1300° F for 30 minutes. A few batches of the foodstuffs have been tested for sodium and chlorine content: sodium by flame photometry after digestion in concentrated nitric

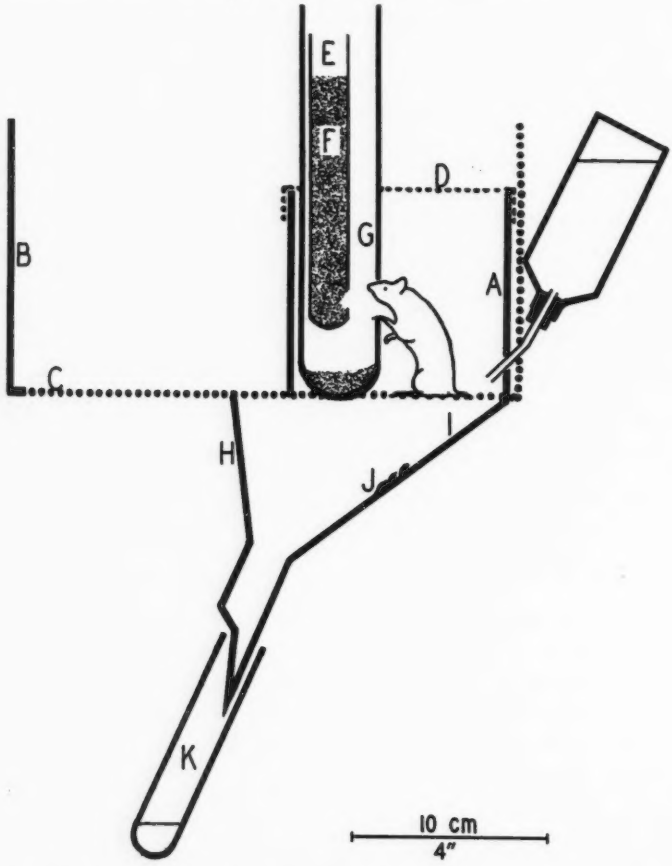

FIG. 1. Mouse cage for urine and feces collection. The cage consists of a cylindrical metal box (A), the bottom of which has been removed. This box is placed in an ordinary metal cage (B) fitted with a wire mesh bottom (C). The top of the box is closed by a wire mesh (D). A glass tube (E) containing the food (F) in powder form is placed in a larger glass tube (G) which collects most of the food spilled. A polyethylene funnel (H), cut obliquely, is placed under the box. A film of silicone grease (I) spread on the surface of the funnel retains the feces (J), hair and the minute amount of food falling down. The urine runs freely into the ashing tube (K) in which 1 ml. 4N Na_2CO_3 had been placed.

acid, and chlorine by the method of Wilson and Ball (3). Only yeast contained measurable amounts of sodium, 0.03–0.13%, and of chlorine 0.01%. Therefore, the concentration of these ions in the diet did not exceed 0.013% Na and 0.001% Cl.

The cages were kept in a room where the temperature was maintained at 80° ± 5° F. The room had no window and the artificial light was automatically turned on from 8:00 A.M. to 8:00 P.M.

Most of the experiments were designed to investigate the *acute* effect of sodium chloride on the metabolism of radioiodine. Each mouse received 10 μc. carrierfree I^{131} subcutaneously and was placed in a 250 ml. beaker fitted with a wire mesh floor allowing the urine to settle at the bottom. The animal had access to distilled water but not to food. Two and a half hours later the mouse was anaesthesized with ether in the beaker. Blood, urine (including bladder content) and various organs were homogenized in 2N NaOH solution. Standards were made for each tissue by adding a known amount of radioiodine to the hydrolysate of the corresponding tissue from an animal that had not been given radioiodine. In all cases 1 ml. of the homogenized solution was poured on a watch glass-supported filter disc (lintine discs, $1\frac{1}{4}''$ diameter, obtained from Johnson and Johnson, Montreal), dried at room temperature and counted with a Geiger-Mueller counter. The counts were corrected for background and expressed in percent of the injected dose of radioiodine.

In these acute experiments, the isotope was injected at the same time as the treatment was administered. Prior to the time of treatment, all the animals had been subjected to the same conditions and presumably had the same average level of I^{127} in blood and tissues. Since the same amount of radioiodine was injected into each animal, the specific activity (I^{131}/I^{127}) of the iodide in the body must have been the same in all groups. Thus the I^{131} content of a tissue truly reflected its I^{127} content, and the experimental production of a change in radioiodine content indicates an actual change in I^{127} metabolism.

In an experiment designed to examine the *chronic* effect of dietary sodium chloride on iodine metabolism, some mice were kept in individual "metabolic cages" (Fig. 1) for measurement of urinary and fecal iodine (I^{127}). The design of the food container was such as to minimize the amount of food falling through the wire mesh floor. A plastic funnel coated with iodine-free silicone grease was held under the cage, so that the feces, as well as hair and the minute amount of food falling through, were held by the grease. The urine ran down the funnel walls into a test tube, where it was collected free of feces, hair and food. The feces were removed from the funnel with forceps. The iodine content of thyroid, urine, and feces was estimated according to a modification of the Barker (4) dry ashing method. In this experiment, other mice, used for I^{131} measurement, were kept in non-metabolic cages. These cages, referred to as "ordinary cages," consisted of galvanized iron drawers, 76×14×14 cm., inserted into a rack. The floor and front wall were made of wire mesh. Food was provided in cold-cream jars.

PROCEDURES AND RESULTS

First hypothesis: sodium chloride enhances the output of thyrotrophic hormone by a direct effect on the pituitary

This hypothesis was tested by administering thiouracil—which is known to induce an excessive secretion of thyrotrophic hormone causing an increase in thyroid weight—to two groups of animals fed diets with and without sodium chloride respectively. If the hypothesis were correct, the thyrotrophic hormone output and, therefore, the thyroid weight would be greater in the presence than in the absence of sodium chloride.

Forty mice with a body weight averaging 18–19 gm. were fed on a batch

of the basic diet containing 6.1 μg. iodine per 100 gm. Half of them received 1%, and the other no sodium chloride in the diet. Both groups were divided into two sub-groups, one receiving no additional treatment, the second 0.2% thiouracil mixed in the diet. Twenty-four days later each mouse was sacrificed with ether and the left thyroid lobe was weighed. Thiouracil increased thyroid weight to about the same extent whether or not sodium chloride was administered (from 0.46 to 2.54 mg. without sodium chloride and from 0.76 to 2.80 mg. with sodium chloride). Thus, no evidence was obtained in favor of the hypothesis that the primary effect of sodium chloride is to enhance the secretion of thyrotrophic hormone from the pituitary.

Since an increased amount of thyrotrophic hormone accelerates the release of radioiodine-labelled hormone from the prelabelled thyroid, another approach was to test whether or not sodium chloride had an effect on this release. Forty mice fed on a batch of the basic diet containing 0.6 μg. iodine per 100 gm. for 3 weeks were given a single subcutaneous injection of 10 μc. carrierfree I^{131}. Twenty-four hours later, 30 mg. of sodium chloride were given by intragastric injection and the animals were sacrificed after 10 and 25 hours. The radioiodine content of the prelabelled glands was not significantly different in controls—59.2 and 51.7% of the dose at 10 and 25 hours respectively—and in sodium chloride treated animals—52.9 and 57.8% at 10 and 25 hours respectively. Thus the salt did not influence the release of hormonal I^{131} from the thyroid. These observations again suggested that the primary effect of sodium chloride was not to enhance the release of thyrotrophic hormone from the pituitary.

Second hypothesis: sodium chloride interferes with iodine metabolism in the thyroid in the same manner as antithyroid drugs

The acute effect of sodium chloride on radioiodine metabolism was compared to that of the antithyroid drug, propylthiouracil. Mice were fed on a batch of the basic diet containing 2.1 μg. iodine per 100 gm. for 3 weeks and were then divided into two groups. The first group received 0.2 ml. of water and the second 30 mg. NaCl in 0.2 ml. of water by intragastric injection. Half of the animals in each group had no further treatment, the other half were injected subcutaneously with 2.5 mg. propylthiouracil (5 mg. of 6N-propylthiouracil per ml. in 0.04N NaOH). Simultaneously, all mice received 10 μc. I^{131} subcutaneously. Two and a half hours later they were sacrificed with ether and the radioiodine content of thyroid, blood and urine was measured.

Propylthiouracil, but not sodium chloride, diminished the thyroid I^{131} uptake (Table 1), whereas sodium chloride, but not propylthiouracil, increased the urinary excretion and lowered the I^{131} blood level. Thus, the two substances had an entirely different effect, and it was concluded that sodium chloride did not act as an antithyroid drug. The incidental observation that sodium chloride increased by 20 times the amount of radioiodine

TABLE 1. THE ACUTE EFFECT OF SODIUM CHLORIDE AND PROPYLTHIOURACIL ON RADIOIODINE METABOLISM IN MICE

Propyl-thiouracil injected, mg.	NaCl injected, mg.	Number of animals	% of injected radioiodine in		
			Thyroid*	1 ml. Blood*	Urine*
0	0	7	10.6 ±0.9	4.3 ±0.4	0.5 ±0.2
0	30	7	10.5 ±1.0	*1.9* ±0.4	*11.6* ±2.6
2.5	0	8	*2.6* ±0.3	6.0 ±0.8	0.8 ±0.3
2.5	30	9	*2.0* ±0.3	3.6 ±0.5	*8.3* ±2.9

* Mean ± standard error of the mean.
The italicized values are significantly different from those found in controls injected with water only.

excreted in the urine, while the thyroidal uptake was left unchanged (Table 1) raised the possibility that this salt depleted the body stores of iodine by promoting its urinary excretion. Hence the next hypothesis.

Third hypothesis: sodium chloride increases the excretion of iodine by the kidney and, as a result, deprives the thyroid gland of iodine

The experiment just described was repeated in bilaterally nephrectomized and in thyroidectomized mice. Thyroidectomy was performed according to a modification of the method of Dent, Gadsden and Furth (5). As soon as the operation had been completed, sodium chloride and radioiodine were injected in the same manner as in previous experiments. The results (Table 2) revealed that sodium chloride increased the urinary excretion of radioiodine and decreased the blood radioiodine in thyroidectomized as well as in control animals. Therefore, these two effects of sodium chloride administration were not mediated by the thyroid gland. However, when the kidneys had been removed, the level of radioiodine in blood was no longer affected by sodium chloride, a result indicating that the lowering of the blood radioiodine produced by this salt in intact animals was due to the increased excretion of radioiodine by the kidney.

TABLE 2. THE ACUTE EFFECT OF SODIUM CHLORIDE ON RADIOIODINE METABOLISM IN THYROIDECTOMIZED AND BILATERALLY NEPHRECTOMIZED MICE

Treatment	NaCl injected, mg.	Number of animals	% of injected radioiodine in		
			Thyroid*	1 ml. Blood*	Urine*
Controls	0	7	10.6 ±0.9	4.3 ±0.4	0.5 ±0.2
	30	7	10.5 ±1.0	*1.9* ±0.4	*11.6* ±2.6
Nephrectomy	0	9	10.8 ±2.0	5.3 ±1.1	—
	30	9	11.4 ±2.2	5.9 ±1.2	—
Thyroidectomy	0	10	(trachea)† 0.13 ±0.025	10.4 ±0.9	1.1 ±0.3
	30	10	*0.07* ±0.007	*7.3* ±1.0	*11.1* ±3.6

* Mean ± standard error of the mean.
The italicized values are significantly different from those found in animals subjected to the same treatment but receiving no sodium chloride.
† The difference in tracheal I^{131} reflects the difference in blood I^{131}.

More information about the sequence of events was obtained by repeating the experiment and sacrificing the animals at two different time intervals, 3 and 24 hours after radioiodine injection. The urinary excretion of radioiodine was increased ten times after 3 hours and 5 times after 24 hours under the influence of sodium chloride (Table 3). The radioiodine content of the tissues was decreased to a greater extent at 24 hours than at 3 hours after sodium chloride administration. The more pronounced depletion of radioiodine in the blood at 24 than at 3 hours under the influence of sodium chloride clearly indicated that the effect of the salt extended well beyond 3 hours.

The thyroidal uptake of radioiodine was only slightly affected by sodium

TABLE 3. THE ACUTE EFFECT OF SODIUM CHLORIDE ON RADIOIODINE METABOLISM IN MICE, AS MEASURED 3 AND 24 HOURS AFTER ADMINISTRATION

Time interval between radio-iodine injection and sacrifice	NaCl injected mg.	Number of animals	% of injected radioiodine in				
			Thyroid*	1 ml. blood*	Urine*	Gastro-intestine*	Carcass*
3 hours	0	8	16.9±1.4	3.5±0.2	2.0±0.5	28.7±3.0	39.3±1.3
	30	9	13.4±1.2	2.4±0.6	*22.0*±4.6	*13.5*±2.4	35.5±1.8
24 hours	0	8	49.7±2.8	1.5±0.3	8.1±3.8	13.5±2.7	21.3±1.9
	30	9	*28.3*±2.5	*0.3*±0.1	*38.4*±5.8	*4.6*±2.3	17.2±3.0

* Mean ± standard error of the mean.
The italicized values are significantly different from those found in animals sacrificed after the same period of time but receiving no sodium chloride.

chloride at 3 hours ($0.05 > P > 0.1$), but was significantly decreased at 24 hours ($P = 0.001$) to about one half the control value. Thus, the decrease in the radioiodine content of the thyroid appeared at a later time after sodium chloride administration than the increase in urinary iodine excretion.

The immediate increase in radioiodine excretion due to sodium chloride and the later occurrence of a decrease in blood and thyroid radioiodine strongly suggested that sodium chloride increases iodine losses and thus reduces the amount of iodine available to the thyroid. A relative iodine deficiency would thus be created and, as a result, the thyroid would eventually become stimulated.

Thyroid stimulation has actually been observed in animals fed sodium chloride in their diet for many weeks (1); the evidence which has been presented concerning the mechanism of the stimulation was obtained in animals receiving a single injection of the salt. It was, therefore, necessary to determine whether or not *chronic administration of sodium chloride*, under conditions which elicit thyroid stimulation, is associated with the same changes in iodine metabolism as were observed after acute administration

of the salt. Mice were divided into two groups, one fed on a batch of the basic diet containing 2.5 μg. iodine per 100 gm. for 2 months, and the other on the same diet containing 1% sodium chloride. Food and distilled water were given ad libitum. Each group was divided into 6 sub-groups.

Information on I^{127} excretion was obtained in those animals of the first sub-group that were housed in individual metabolic cages (Fig. 1); urine and feces were collected once a week over a 24 hour period and their I^{127} content was measured. The food intake was estimated at intervals. Sixty-three days after the beginning of the experiments, the animals were sacrificed, the weight and the I^{127} content of one lobe of the thyroid was measured, and the other lobe was examined histologically.

Information on I^{131} uptake by the thyroids and excretion in the urine was obtained in the other 5 sub-groups, which were housed in ordinary cages

TABLE 4. THE EFFECT OF DIETARY SODIUM CHLORIDE ON THE THYROID OF MICE FED ON A LOW IODINE DIET FOR 63 DAYS

Housing	% NaCl in diet	Number of animals	Body weight,* gm.	Thyroid weight,* mg.	Thyroid I^{127}* μg.
Metabolic cages†	0	8	27.6 ± 1.5	3.3 ± 0.3	1.06 ± 0.28
	1	8	27.4 ± 0.6	5.6 ± 0.4	0.26 ± 0.24
Ordinary cages	0	11	23.1 ± 1.3	2.2 ± 0.3	1.38 ± 0.19
	1	11	22.6 ± 0.9	2.5 ± 0.2	1.33 ± 0.29

* Mean ± standard error of the mean.
† In the animals housed in the metabolic cages, the weight and iodine content of the thyroid have been measured using the left lobe only. The figures obtained were doubled for presentation in this table.

(6–8 animals per cage). These animals were sacrificed after 1, 7, 21, 35 and 63 days respectively. On the evening preceding sacrifice, food was removed from their cages. Two and a half hours before sacrifice, the mice were given access for half an hour to the same diet as before but labelled with 10 μc. I^{131}/gm..diet. After one half hour, during which each animal ate an average of 0.37 gm. of food, it was placed individually in a 250 ml. beaker for urine collection, as in the acute experiments, and was sacrificed 2 hours later. I^{131} was measured in urine and thyroid. In the last sub-group, sacrificed at 63 days, the weight and the I^{127} content of the thyroid were also measured.

The animals kept in metabolic cages, whether fed sodium chloride or not, had goiters which histologically showed the usual signs of thyroid stimulation. Thyroid weights were higher in the sodium chloride treated than in the control group (Table 4). Histologically, although individual variations were large, there seemed to be on the average less colloid in sodium chloride fed than in control animals. Furthermore, the total iodine content of the thyroid was diminished by sodium chloride (Table 4, last column). The mice receiving sodium chloride excreted more iodine in the urine than controls did (Table 5, Fig. 2), except during the last two weeks,

TABLE 5. THE EFFECT OF DIETARY SODIUM CHLORIDE ON IODINE METABOLISM IN MICE

Days on diet	% NaCl in diet	mμg. I^{127}* excreted daily (mice in metabolic cages)		Radioiodine distribution* (mice in ordinary cages)		mμg. I^{127}† newly accumulated in thyroid
		Urine	Feces	Thyroid I^{131}.100 / Thyr. I^{131}+Ur. I^{131}	Urine I^{131}.100 / Thyr. I^{131}+Ur. I^{131}	
1	0	383 ±56	952 ±386	47.7 ±4.6	52.3 ±3.6	349
	1	248 ±60	996 ±277	*20.3 ±3.2*	*79.7 ±3.2*	63
7	0	221 ±32	233 ± 52	39.4 ±5.5	60.6 ±5.5	144
	1	*431 ±92*	260 ± 26	*13.8 ±1.5*	*86.2 ±1.5*	71
14	0	88 ±17	212 ± 36	—	—	—
	1	108 ±17	166 ± 37	—	—	—
21	0	44 ± 5	117 ± 16	37.7 ±4.7	62.3 ±4.7	27
	1	56 ± 6	120 ± 28	*23.4 ±3.8*	*76.6 ±3.8*	17
28	0	12 ± 4	118 ± 7	—	—	—
	1	*32 ± 5*	*83 ± 11*	—	—	—
35	0	19 ± 5	96 ± 12	72.2 ±5.8	27.8 ±5.8	49
	1	23 ± 6	*61 ± 7*	*39.3 ±9.1*	*60.7 ±9.1*	15
42	0	5 ± 1.7	102 ± 21	—	—	—
	1	13 ± 4.6	71 ± 7	—	—	—
49	0	6.6 ± 2.8	91 ± 21	—	—	—
	1	4.9 ± 2.2	55 ± 9	—	—	—
56	0	4.5 ± 1.3	50 ± 4	—	—	—
	1	4.2 ± 1.6	*66 ± 6*	—	—	—
63	0	6.1 ± 2.6	57 ± 9	88.5 ±2.6	11.5 ±2.6	47
	1	3.9 ± 1.7	39 ± 10	*75.1 ±5.0*	*24.9 ±5.0*	12

* Mean ± standard error of the mean.

$$\dagger = \text{Urine } I^{127} \cdot \frac{\text{Thyroid } I^{131}/(\text{Thyr. } I^{131} + \text{Ur. } I^{131})}{\text{Urine } I^{131}/(\text{Thyr. } I^{131} + \text{Ur. } I^{131})}$$

The values showing a significant effect of sodium chloride are italicized.

when the amount of iodine excreted was too small to permit significant differences to be detected by the technique. In feces, iodine was excreted in the same amount in the two groups during the first three weeks; but less iodine was found in the feces of sodium chloride fed than of control animals during the next four weeks (Table 5, Fig. 3). At the end of the experiment the total amount of iodine excreted in feces and urine, about 0.06 μg. per day, was close to the amount of iodine ingested, 0.07 ±0.015 μg. per day (as calculated from the daily food intake, 2.9 ±0.2 gm., and the iodine content of the diet, 2.5 ±0.5 μg. per 100 gm.). In the early phase of the experiment the urinary excretion accounted for an important fraction of the total iodine losses; later on this fraction became gradually smaller, and, at the end, was only about 10% of the total (Table 5).

The thyroid glands of the mice housed in ordinary cages were not significantly increased in weight by sodium chloride and were, in fact, smaller

than in the mice kept in metabolic cages (Table 4). Furthermore, the mice in ordinary cages lost 0.3 gm. whereas those in metabolic cages gained 3.7 gm. on the average during the course of the experiment. (The uncontrolled factors that interfered with the growth of the mice housed in ordinary cages may also account for the smaller thyroid weights). Nevertheless radioiodine measurements showed that striking differences in iodine metabolism were brought about by sodium chloride. The computation of radioiodine data

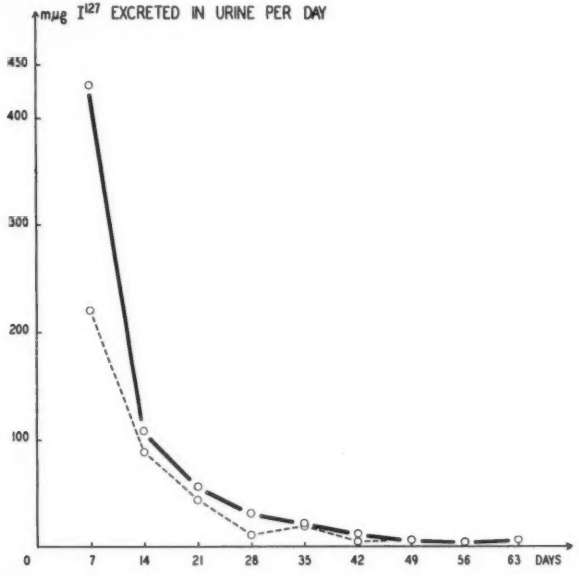

FIG. 2. Effect of dietary sodium chloride on the urinary excretion of iodine in mice fed on a low iodine diet (mice in metabolic cages). Sodium chloride increased the urinary iodine.

required a rather elaborate method since the animals ate variable amounts of I^{131}-labelled food and, as a result, the dose of radioiodine ingested varied from mouse to mouse. Under these conditions, it was unsafe to average the radioiodine contents of tissues or excreta and to compare them in sodium chloride fed and control mice, but it was safe to compare the I^{131} content of the thyroid to that of the urine in any mouse. This was done in each animal by calculating the ratios:

$$\frac{\text{Thyroid } I^{131} \cdot 100}{\text{Thyroid } I^{131} + \text{Urine } I^{131}} \quad \text{and} \quad \frac{\text{Urine } I^{131} \cdot 100}{\text{Thyroid } I^{131} + \text{Urine } I^{131}}.$$

The results (Table 5, Fig. 4) showed that the radioiodine distribution was drastically modified in the animals given sodium chloride over a long period of time. Under the influence of this salt the fraction of the radioiodine lost

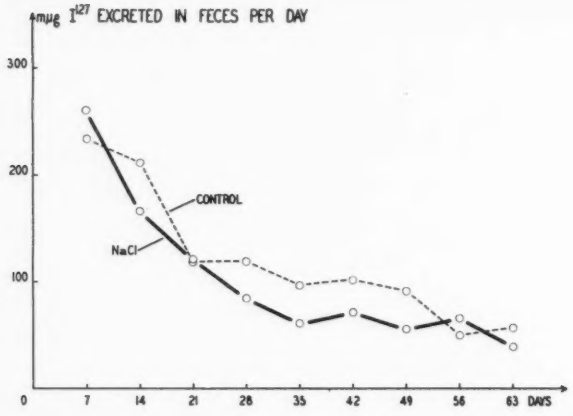

FIG. 3. Effect of dietary sodium chloride on the fecal excretion of iodine in mice fed on a low iodine diet (mice in metabolic cages). Sodium chloride decreased the fecal iodine.

in urine was increased and that taken up by the thyroid was decreased; the proportion retained in the thyroid was cut to one half of the control value at the beginning, but only to five sixths of the control value at the end of the experiment. Apparently, as the thyroid became more active, the effect of sodium chloride on the urinary excretion of iodine decreased.

Since data were available for the relative distribution of iodine between thyroid and urine (mice in ordinary cages, Table 5) as well as the absolute amount of I^{127} excreted daily in the urine (mice in metabolic cages, Table

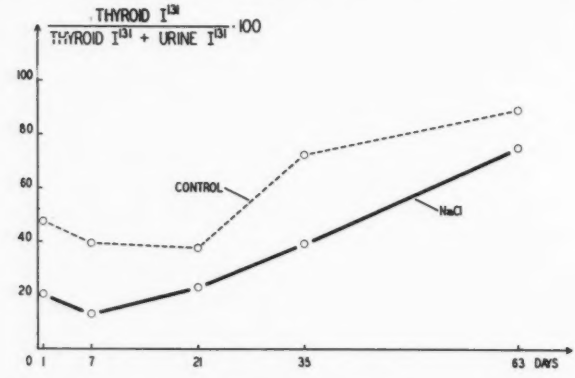

FIG. 4. Effect of dietary sodium chloride on the radioiodine uptake of the thyroid expressed as percentage of the total radioiodide concentrated in both thyroid and urine. (The complement to 100 is lost in the urine.)

If it is assumed that all the iodide in the body ultimately goes either to the thyroid or the urine, the ratio indicates the proportion of the body iodide diverted to the thyroid. It thus appears that the thyroid share of the body iodide is decreased by sodium chloride.

5) it was possible to calculate the amount of I^{127} newly accumulated in the thyroid per day (Table 5, last column). (This calculation assumes that the body inorganic iodine ultimately goes either to the thyroid or into the urine). At the end of the experiment the thyroid weights in the mice used for I^{127} estimation (metabolic cages) were different from those in the mice used for the determination of the relative distribution of iodine (ordinary cages) (Table 4); the thyroid glands must therefore have gradually become different under the two different conditions of housing. Thus only the earlier values in the last column of Table 5 can be considered reliable. Examination of these values shows that the amount of I^{127} accumulated daily in the thyroid was markedly reduced by prolonged ingestion of sodium chloride.

DISCUSSION

The histological picture of hypertrophy, hyperplasia, hyperemia and colloid loss observed in the thyroid gland of the mouse after prolonged ingestion of sodium chloride was identical with that resulting from chronic exposure to cold or to antithyroid drugs. In both of these conditions, it is known that the level of circulating thyrotrophic hormone is raised (6, 7) and in fact, the picture was similar to that obtained soon after injection of thyrotrophic hormone itself. Accordingly, even though no direct measurement of the circulating level of thyrotrophic hormone was made, it was reasonable to conclude that ingestion of sodium chloride ultimately resulted in an increased secretion of this hormone from the pituitary. However, the mechanism by which an over-secretion of thyrotrophic hormone was induced in sodium chloride fed animals remained to be elucidated.

Three hypotheses were examined: 1) a direct effect on the *pituitary* enhancing thyrotrophic hormone release; this possibility was eliminated because, unlike thyrotrophic hormone, sodium chloride did not speed up the discharge of thyroidal iodine, nor did it increase the effect of thiouracil treatment; 2) a direct effect on the *thyroid gland* by which sodium chloride would, like thiourea, inhibit the incorporation of iodine into thyroglobulin, or, like thiocyanate, impair the concentration gradient of iodide between thyroid and blood (8); either possibility should produce an immediate, profound depression of radioiodine uptake (9), but such an effect was not observed (Table 1); 3) the production of a relative iodine deficiency by an increase in the *urinary excretion of iodine*, which was actually shown to happen after *acute* administration of sodium chloride (Tables 1–3). Measurements of the clearance of radioiodide between the 3rd and 24th hour after administration of 30 mg. of sodium chloride[4] revealed that 13 microliters of blood were then cleared per minute, as compared with only 2 microliters in controls. Thus a 6.5 fold increase in the renal clearance of iodide was caused

[4] The radioiodine in blood was assumed to be inorganic only. The clearances were calculated from the radioiodine content of blood and urine at 3 and 24 hours after in-

by the salt (a result in agreement with observations made in the dog, 10). Since the increase in the urinary excretion of radioiodide under the influence of sodium chloride was observed in thyroidectomized animals (Table 2), this phenomenon must have occurred independently of the thyroid gland. Furthermore, the lowering of the blood radioiodide level after sodium chloride administration (Table 1) was not observed in the absence of the kidney (Table 2); it was, therefore, attributed to the urinary loss of radioiodide. Finally, sodium chloride had no significant effect on the radioiodine content of the thyroid measured after 3 hours, but decreased the 24 hour uptake (Table 3). The three hour result was in line with the earlier findings that sodium chloride did not impair the ability of the thyroid to take up iodine (Table 1). Since it was shown earlier than the salt did not influence the release of radioiodine from the gland either, the low thyroid I^{131} at 24 hours could only be explained by a decrease in the availability of radioiodine. The overall sequence of events appears to be that sodium chloride increases the urinary excretion of radioiodine and, as a result, lowers the circulating level of iodide so that less iodide is made available to the thyroid gland.

This conclusion was derived from experiments in which a single, acute administration of sodium chloride was used, but a similar sequence of events was observed in animals *chronically fed on the diet containing 1% sodium chloride*. At the end of the first week, these animals started excreting more iodine (I^{127}) in the urine than did the controls (Fig. 2). Similarly, calculations of the urinary excretion of administered radioiodine[5] indicated

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jection (Table 3) over the interval of 21 hours (1260 minutes) according to the formula:

$$(U_{24} - U_3) \cdot \frac{\ln B_{24} - \ln B_3}{B_{24} - B_3} \cdot \frac{1}{1260} \text{ ml. cleared per minute}$$

$U_3 = I^{131}$ excreted in the urine during the first 3 hours (% of injected dose)
$U_{24} = I^{131}$ excreted in the urine during the first 24 hours (% of injected dose)
$B_3 = I^{131}$ concentration in blood at 3 hours (% of injected dose/ml.)
$B_{24} = I^{131}$ concentration in blood at 24 hours (% of injected dose/ml.)

[5] On the basis of the results reported in Table 3, it was assumed that at the beginning of the experiment the 2 hour radioiodine uptake of the thyroid was the same whether or not the animals ingested sodium chloride. As long as this is the case, it is readily seen that:

$$\frac{\text{Urinary } I^{131} \text{ in NaCl group}}{\text{Urinary } I^{131} \text{ in Control group}} = \frac{U_{NaCl}}{U_{Controls}} \cdot \frac{T_{Controls}}{T_{NaCl}} \qquad (1)$$

in which

$$T = \frac{\text{Thyroid } I^{131}}{\text{Thyroid } I^{131} + \text{Urine } I^{131}} \qquad U = \frac{\text{Urine } I^{131}}{\text{Thyroid } I^{131} + \text{Urine } I^{131}}$$

The formula (1) was used to compare the excretion of radioiodide in sodium chloride fed and control animals, and showed that, after one day treatment, the sodium chloride group excreted 3.6 times more, and after 7 days, 4.0 times more radioiodine than the controls.

a 4-fold increase over that of controls. Presumably the renal clearance had also increased four times, since it is likely that the circulating level of radioiodine was not much affected by sodium chloride under the conditions used. (It was reasoned that since an 11-fold increase in urinary excretion lowered the blood radioiodide by only 30%, as shown in Table 3, the 4-fold increase found here should have had still less effect on the blood radioiodide.)

The plasma level of inorganic iodine (I^{127}) in animals on a low iodine diet was known to become too low for accurate estimation, so that information on the subject had to be obtained by a circuitous route. Although the renal clearance was increased 4 times in the sodium chloride fed animals, the urinary I^{127} was increased only 1.8 times on the average during the first 6 weeks (Table 5). Therefore, the plasma iodide (I^{127}) level in the mice fed the salt diet must have been about half the level in controls. Thus, under chronic conditions too, sodium chloride increased the renal clearance and decreased the blood level of iodide.

In the thyroid gland less iodine was accumulated daily (Table 5, last column) and ultimately less iodine (I^{127}) was found in sodium chloride fed than in control animals (Table 4), a result in agreement with the observation of Remington (11) that, in rats fed a low iodine diet containing 1% sodium chloride, the iodine content of the thyroid gland was 0.8 μg. as compared to 2.5 μg. in animals fed a diet free of the salt. Indeed, the increase in radioiodine excretion and thyroid weight caused by sodium chloride in the mouse was also found to occur in the rat (Isler, unpublished).

A smaller thyroid iodine uptake and content must result in a smaller release of thyroid hormone from the thyroid, and consequently a lower level of circulating hormone in sodium chloride fed than in control animals. An index of the amount of hormone released from the thyroid gland could be derived from the quantity of iodine (I^{127}) excreted via the feces, since the fecal iodine consists almost exclusively of thyroxine and triiodothyronine (12) and its amount is related to the dose of thyroxine administered (13). The smaller amount of fecal iodine found in sodium chloride treated mice (Table 5) suggests that such animals released less thyroid hormone into the circulation than did the controls. The reduced level of this hormone in blood would result in an increased pituitary secretion of thyrotrophic hormone which, in turn, would account for the ultimate increase in thyroid weight.

In conclusion, the data obtained support the concept that the stimulation of the thyroid gland observed after prolonged ingestion of sodium chloride occurs according to the following mechanism:

1) The urinary excretion of iodide is increased, and, as a result, the blood level of iodide is decreased, so that the amount of iodide taken up by the thyroid is reduced.

2) Consequently, the amount of hormone produced and released by the thyroid gland is diminished, and the level of circulating thyroid hormone is reduced.
3) As a result, the pituitary gland increases its secretion of thyrotrophic hormone, which eventually stimulates the thyroid gland.

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AN IMPROVED PROCEDURE FOR THE BIOLOGICAL ASSAY OF ANDROGENS BY DIRECT APPLICATION TO THE COMBS OF BABY CHICKS[1]

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ABSTRACT

Experiments on improvement of the biological assay method for androgens by direct application to the combs of baby chicks led to five successive modifications in procedure, each of which was put into practice and evaluated for precision, minimum effective dose (m.e.d.), and convenience. A total of 162 assays and experiments using approximately 20,000 baby chicks was involved.

The response metameter, the readily computed

$$\log \frac{\text{comb weight}}{\text{body weight}},$$

was selected after testing several other transformations of the data. It yielded a linear log dose-response curve, minimized the variance, and was homoscedastic.

In other experiments it was shown (1) that when the volume of solution applied to the comb daily was reduced from 0.05 ml. to 0.01 ml., the m.e.d. was lowered markedly, (2) that absolute ethanol was substantially as effective (at a volume of 0.01 ml.) as well as more convenient than ether as the vehicle for application of androgen, (3) that 7 daily applications produced more precise results than 5 applications, and (4) that there was a hump at the lower end of the log dose-response curve that could be avoided, thus improving the precision of the assay.

The procedure for the assay currently in use in our laboratory (the fifth modification) is described in detail. The results of 29 assays in this series were evaluated statistically and compared with the results of the previous modifications. The m.e.d. was less than 0.05 μg. androsterone per chick per day. The mean index of precision (λ) was 0.34, with a standard deviation of 0.056. With 40 chicks on the standard and 20 on each unknown the expected minimum % standard error of a potency estimate was 24%. There was much less interassay error than in the first three modifications of the assay method.

THE classical method of Gallagher and Koch (1, 2) for the biological assay of androgens, based on the growth of the capon's comb follow-

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ing intramuscular injections of androgen in oil, is probably unsurpassed in precision. The method has certain disadvantages however, mainly the inconvenience and expense of maintaining a colony of capons and the relatively large doses of androgen required to produce a significant effect. Johnston (3) found that direct application of androgen in ether solution to the comb of the capon markedly reduced the minimum effective dose (m.e.d.); however, the precision of this modification was much lower than that of the original method. The use of the baby chick, a more convenient and economical assay animal, for the quantitative assay of androgens was introduced by Frank *et al.* (4–6). In their method the androgen was applied in oil solution directly to the comb, and the effect was determined from the weight of the excised comb on the day following the last application of androgen. The baby chick method has also been studied and used extensively by Dorfman *et al.* (7–11). The starting point for the present investigation was the baby chick method of Rakoff *et al.* (12), who, like Johnston, reduced the m.e.d. greatly by applying the androgen in ether solution rather than in oil. The low m.e.d. was virtually essential for certain studies in which we wished to assay the androgenic activity of extracts of urine from adrenalectomized, orchiectomized elderly patients with prostatic carcinoma (13–15) and in the assay of steroids of very low androgenic potency.

Numerous experiments directed toward further improvement of the assay method have been conducted in our laboratory over the past five years. Five successive modifications in procedure grew out of these experiments, and each one was put into practice for a period of time sufficient to permit conservative evaluation of its precision, minimum effective dose, and convenience. A total of 162 assays and experiments using approximately 20,000 baby chicks were involved.

The present report consists of a summary of our experiments, a detailed description of the improved assay procedure currently in use in our laboratory, and statistical data on precision and reliability based on extensive experience with each modification of the assay method.

MATERIALS AND METHODS

Single Comb White Leghorn cockerels were shipped by the supplier[2] on the day of hatching. This breed has been shown by Dorfman (9) to be the most suitable for use in the assay of androgens. After arrival in the laboratory on the following day the chicks were distributed randomly into metal cages, 11 by 22 by 12 inches, with removable wire mesh floors. The cages were located in an air-conditioned animal room with temperature regulation. Additional heat was provided by a 25-watt lamp in each cage. The feed was supplied in long, narrow metal troughs and water was provided in large open dishes with reservoir bottles. All the chicks in each cage received the same treatment.

Original Procedure Used for the Assay. In each assay there were 100–110 chicks, divided into 10 equal groups, one group to a cage. The reference standard, androsterone, was applied in 0.05 ml. of ether directly to the comb at dose levels of 0.1, 0.4, and 1.6 μg.

[2] Hall Brothers Hatchery, Wallingford, Conn.

per day, one dose level to each of three groups. A control group was given ether alone, 0.05 ml. daily. The remaining chicks were used for testing unknowns. The treatments were continued daily for seven days, beginning on the second or third day of life. On the morning following the last day of treatment and after an overnight fast, the chicks were killed, weighed, and the combs excised and weighed.

Development of the Assay Procedure through Five Modifications. The principal distinguishing features of the five modifications are shown in the first part of Table 1. The statistics[3] in the latter part of the table show the effect, if any, of each modification on the precision of the results. The current assay procedure (Series V) and its statistical characteristics are described in more detail in a later part of this paper.

EXPERIMENTS ON IMPROVEMENT OF THE ASSAY METHOD

Choice of the Response Metameter. The fact that the variance of the comb weights of chicks treated with androgen increases with increasing response

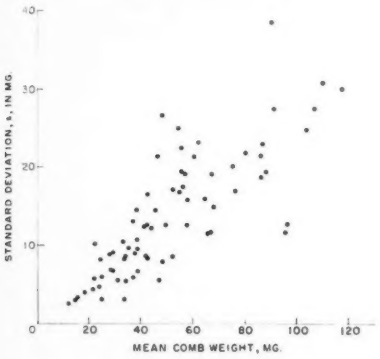

FIG. 1. Standard deviation plotted against mean comb weight of 73 groups of chicks in 7 androgen assays, showing increasing variation with increasing response when the unadjusted comb weights were used.

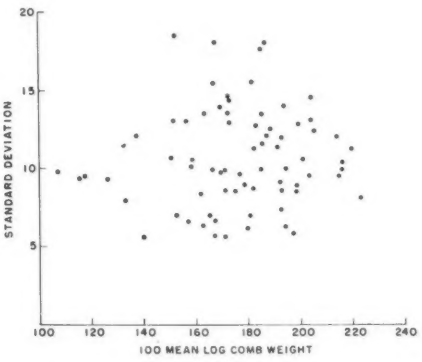

FIG. 2. Standard deviation plotted against mean log comb weight of the same groups of chicks as in Fig. 1, showing variation independent of response when the comb weights were transformed into logarithms.

has been reported by some investigators (16, 17), may be observed in examining other published data, and has been confirmed by our own experience, as illustrated by the data plotted in Figure 1. Since this phenomenon renders invalid the usual formulas used in the computation of assays, it is necessary to consider a corrective adjustment such as transformation of the response metameter. Transformation of the comb weights to their logarithms brought the heteroscedasticity (inequality of variance) below the P .05 level of significance and removed the association between variation and size of the comb, as shown in Figure 2.

The originators of the chick assay method incorporated an adjustment for the observed correlation between the comb weight and the body weight into the response metameter (18). We have re-examined this relationship

[3] The statistics are based on a total of 139 routine assays; 19 experiments and 4 grossly invalid assays were not included in the calculations.

in an attempt to minimize the residual variance of the assays. Neither the initial body weight of the chicks nor the gain in body weight during the treatment was found to be a significant factor in the response of the comb. There was, however, a correlation between the log comb weight and the body weight or log body weight at autopsy. In 8 out of a sample of 11 assays, the slope of regression of log comb weight on log body weight lay between 0.9 and 1.2. Since a slope of 1.0 implies that the ratio

$$\frac{\text{comb weight}}{\text{body weight}}$$

is a constant, it was considered justifiable to use the simple log of this ratio as the response metameter rather than the computationally more laborious covariance adjustment. In 29 out of a sample of 32 assays, the use of the log ratio resulted in decreased variance as compared with the log comb weight alone. Values obtained with the log ratio were not appreciably inferior to those obtained by covariance adjustments, or by analyses of the

$$\log \frac{\text{comb weight}}{(\text{body weight})^2} .$$

Since the conditions for valid biological assays (19) include a Normal Distribution of the response metameter, the log ratio was tested for its agreement with this specification. The deviation of each value from its treatment group mean was computed for the 1340 values obtained in assays 157–162. The size of the deviations was not related to the magnitude of the mean. Positive and negative deviations were equally common and essentially of the same size. The only significant ($P < .01$) departure from a Normal Distribution consisted of leptokurtosis, i.e. the curve showed a high peak and long tails. However, the type and degree of non-normality observed were not sufficient to affect assay estimates appreciably.

Finally, it was found, in agreement with an observation made earlier by Dorfman (8), that the simple ratio

$$\frac{\text{comb weight}}{\text{body weight}}$$

resulted in nonlinear dose-response curves that became linear after the log transformation. In order to ensure linearity, however, the lower portion of the dose-response curve must be avoided, as shown later in this report.

The Effect of the Volume of Solution Applied Daily. Previous investigators (20–23) had observed that a given amount of androgen in oil or alcohol solution was more effective when applied in a volume less than 0.05 ml. It could be seen readily in the assays of Series I that even with a solvent as volatile as ether a considerable portion of the daily dose flowed off the comb of the chick onto the feathers during the application of the 0.05 ml.

of solution. Experiments to determine the effect of reducing the volume of ether demonstrated, as shown in Figure 3, that at all dose levels, application in 0.01 ml. of ether was significantly ($P < .05$) more effective than in 0.05 ml. by a ratio of approximately 3.5. The daily applications were therefore reduced to 0.01 ml. of ether solution and the doses of standard androsterone were halved. In all 60 assays providing a comparison, the mean comb response of the chicks on 0.05 μg. of androsterone was higher than the control, and the differences were significant ($P < .05$) in 29 assays (24).

The Effect of Duration of Treatment. The growth of treated and untreated combs during the first 10 days of life was studied in a 2×3 factorial experiment (25). A randomized block design was used in which 12 cages were divided into 2 blocks consisting of the outer and inner cages, respectively.

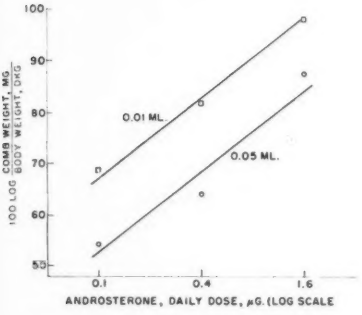

FIG. 3. Effect of the volume of androsterone solution applied daily on the response of the comb.

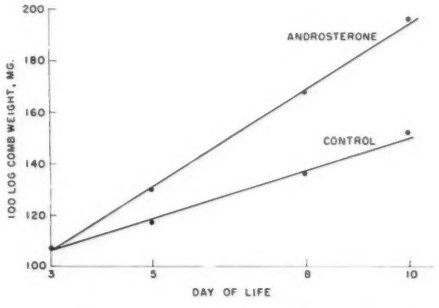

FIG. 4. Increase in comb weight with age and duration of treatment with androsterone (0.8 μg./day).

Daily applications of 0.8 μg. of androsterone in 0.01 ml. of ether were started on the third day of life for 48 chicks, while an equal number received no treatment. One-third of the chicks in each of these groups were autopsied on the fifth, eighth, and tenth day of life, respectively. Eight chicks were assigned at random to each of the six treatments in a randomly selected cage in each block. In addition, 16 untreated chicks were autopsied on day 3 of life.

Both the comb and the total body weight showed a curvilinear growth with time. The stimulation of comb growth by androsterone was already significant ($P < .001$) on day 5, after two treatments, but became progressively more marked at the subsequent time intervals. There was no significant difference between the outer and inner cages. The log comb weights are plotted against time in Figure 4 and show linear regressions with a significant difference in slope between the treated and untreated chicks.

Although the results of this experiment could not determine the optimum duration of androgen treatment for assay purposes, they did suggest that the effect of androsterone after five daily applications was sufficiently

marked to justify a trial of this period of treatment. Eight consecutive assays were conducted according to this plan in Series III, with the unsatisfactory results shown in Table 1.

The effect of postponing autopsy until the second or third day after the last (seventh) application was also studied. There were some indications that this might result in an increased slope. However, prolongation of the assay is inconvenient, and is impractical because the rapid growth and the appearance of aggressiveness in the chicks as they grow older result in overcrowding and injuries.

The Vehicle for Topical Application of the Androgen. The use of ether as the vehicle in which the androgen was dissolved was troublesome because

TABLE 1. PROCEDURAL AND STATISTICAL CHARACTERISTICS* OF FIVE SUCCESSIVE MODIFICATIONS IN DESIGN AND TECHNIQUE OF THE ANDROGEN ASSAY METHOD

Assay modification	I	II	III	IV	V
No. of assays	13	61	8	28	29
Vehicle for androgen	Ether	Ether	Ether	Ethanol	Ethanol
Volume of solution applied daily	0.05 ml.	0.01 ml.	0.01 ml.	0.01 ml.	0.01 ml.
Duration of treatment	7 days	7 days	5 days	7 days	7 days
No. of dose levels of standard	3	3	2	2	2
Range in daily dose of standard	0.1–1.6 μg.	0.05–0.8 μg.	0.05–0.8 μg.	0.05–0.8 μg.	0.1–0.8 μg.
Approximate number of chicks per assay	100	100	100	200	200
per standard (N_S)	30	30	40	40	40
Mean residual variance ($\bar{s}^2$)	105	118	74	95	99
Mean slope of response to standard ($\bar{b}_S$)	24.2	24.2	14.2	24.2	29.5
Mean index of precision ($\lambda = \bar{s}/\bar{b}_S$)	0.42	0.45	0.60	0.40	0.34
Interassay variance of b_S	36.6	30.0	18.1	15.4	16.9
Mean predicted variance of b_S	15	16	5	7	12
Expected minimum V(M)	0.0146	0.0168	0.0270	0.0120	0.0087
Expected minimum % s.e.$_R$	32	34	46	28	24
Expected minimum ratio R_U/R_L at 95% confidence limits	3.0	3.2	4.4	2.7	2.3

* The statistics are based on the response metameter

$$100 \log \frac{\text{comb weight, mg.}}{\text{body weight, dkg.}}.$$

V(M) Variance (V) of the log potency estimate (M).
% s.e.$_R$ Standard error (s.e.) of the potency (R) in %.
R_U, R_L Upper and lower confidence limits for R.

of the difficulty of storing the solutions for the necessary 7-day period without loss of solvent by evaporation. Absolute ethanol was considered as a possible alternative to ether (26, 27). Yet alcohol solutions of androsterone were much less effective in stimulating comb growth than ether solutions containing the same concentration of steroid when the volume applied daily was 0.05 ml. per chick in both cases (Fig. 5). However, when this volume was reduced to 0.01 ml., the difference in effect on comb growth between alcohol solutions and ether solutions was relatively small (Fig. 5). Accordingly, beginning with the assays of Series IV, alcohol was adopted as the solvent for application of androgens.

Following the change in vehicle, the mean residual variance ($\bar{s}^2$) was decreased approximately 20%, from 118 in Series II to 95 in Series IV, and an associated slight improvement in the index of precision (λ) was noted (Table 1). A much more striking and important improvement in the assays of Series IV was the decrease in *interassay error*, which is discussed more

fully under a separate heading. Alcohol is so much more convenient than ether as the vehicle for application of androgen to the combs of chicks that its adoption for the routine assay procedure would have been well justified even if there had been no improvement in the statistical results.

Frequency of Applications. With 0.01 ml. of ethanol as the vehicle, and a total dose of 0.35 or 5.6 μg. of standard androsterone (equivalent to 0.05 μg. and 0.80 μg. per day respectively), the effects of modifications in frequency of applications were studied. When the total dose was divided into fewer applications made on alternate days instead of daily, it appeared to

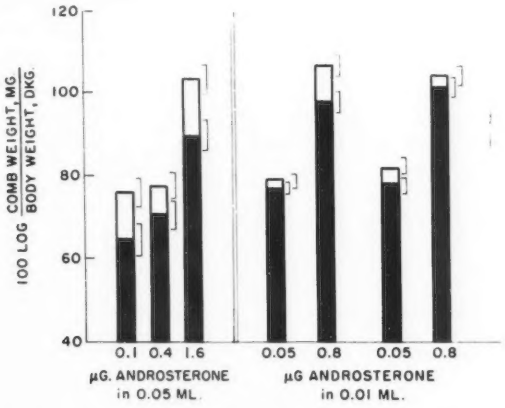

FIG. 5. Comparison of ether and ethanol as vehicles for topical application of androsterone to the comb. The effect of androsterone in ethanol is shown by the height of the solid portion of the bar; the effect in ether by the total height of the bar. The brackets represent the standard errors. When administered in a volume of 0.05 ml. the difference between vehicles was significant; in a volume of 0.01 ml. the difference was not significant.

be somewhat less effective, although in only one of three experiments was the difference significant. After two daily applications the comb growth was not greater than when the same total dose was applied once daily. Accordingly, no change was made in the routine procedure of daily applications.

The Shape of the Log Dose-Response Curve of Androsterone. In Series I, where the reference standard androsterone was administered at three dose levels, 0.1, 0.4, and 1.6 μg. daily, significant nonlinearity appeared in only one assay out of 13. However, the reduction of these dose levels by one-half in Series II was associated with significant curvature at P .05 in 18 out of 61 assays. It was therefore important to examine the shape of the log dose-response curve more critically.

In Figure 6 (left) are shown the results of two experiments in which five dose levels of androsterone in ether were given daily for five days. The results of two similar experiments in which nine dose levels of androsterone in alcohol were given daily for seven days are shown in Figure 6 (right).

Each point represents the mean of two groups of 10–12 chicks treated alike. In both sets of experiments the response was linear to log dose above the dose of 0.1 μg., but there was significant curvature when the results from the 0.05 μg. dose were included. Additional experiments not shown here were consistent with this observation that there is a "hump" in the log dose-response curve below the dose of 0.1 μg.

Accordingly, the low dose of the reference standard was increased from 0.05 μg. to 0.1 μg. The mean slope of the standard was thereby increased

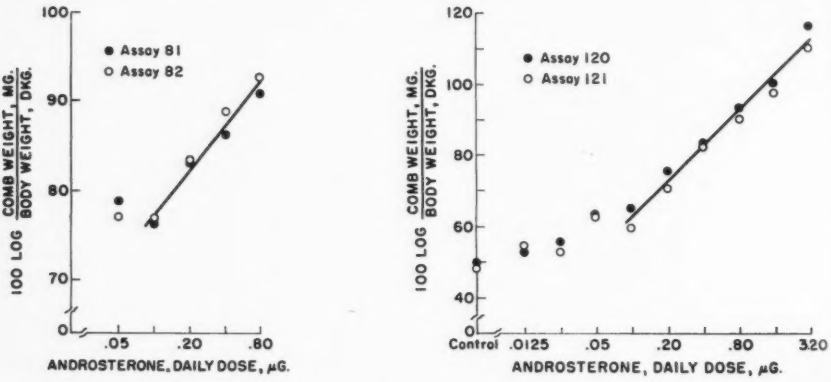

FIG. 6. Log dose-response curves for androsterone. The graph on the left shows the effects of five dose levels in two consecutive experiments when the duration of treatment was five days. The graph on the right shows the effects of nine dose levels in two separate experiments when the duration of treatment was seven days. Each point represents the mean response of 20–24 chicks. In each case the line represents the linear portion of the curve.

from 24.2 in Series IV to 29.5, and the mean index of precision (λ) was improved by 15%, from 0.40 to 0.34.

The increase in the predicted variance of b_S seen in Table 1 was the result expected by a change in the log interval between the low and the high dose of standard from 1.204 in Series IV to 0.903 in Series V, reducing Σx^2 from 14.4 to 8.2 and increasing the quotient $s^2/\Sigma x^2$ correspondingly. However, the observed variance of b_S did not change significantly.

Intercage Variation. Although the results have been analyzed in terms of a single comb response, the assay procedure actually treats a cageful of chicks as the experimental unit. This fact coupled with the omission of full replication of treatments over cages raises questions as to the possible effect of cage differences on the final results. Generally, differences between cages of experimental subjects treated alike are greater than the differences observed among subjects within a cage (28). Although cage effects were randomized in each assay, they might have biased the results of a single assay or inflated the real error between replicate determinations.

Out of eight experiments in which all treatments were duplicated, one showed significant ($P < .05$) cage variation. Assay variation was added to cage variation in an experiment where 12 dose-treatments were tested in four assays (146–9), with independent random assignment of treatments to cages. There was no evidence of cage or assay differences in this experiment (29). However, in our total experience significant differences (at $P < .05$) did occur in 15 out of 81 assays (19%) where some replication had been provided. This incidence is greater than one would expect by chance ($P < .01$).

It is impractical to control intercage error by assigning a different treatment to each chick in a cage. A more feasible alternative would be to replicate cages within each assay for all treatments. However, we have considered it preferable to spread the limited number of chicks available for each unknown over as many independent assays as possible, thus giving consideration to intercage error and interassay error simultaneously. In the current assay procedure the replication of standards in every assay provides a certain degree of continuous control over the occurrence of intercage error and the opportunity to correct various details of technique which may have contributed to it.

CURRENT PROCEDURE FOR THE BIOASSAY (SERIES V)

In each assay there are 210–230 chicks, divided into 20 groups of approximately equal size. Four groups of chicks are given the reference standard, androsterone, two groups at the dose level of 0.1 μg/day and two at 0.8 μg./day. There are usually one or two control groups which are given 0.01 ml./day of absolute ethanol only. The remaining 14 or 15 groups are used for testing unknowns. The cages and corresponding groups of chicks are numbered consecutively, and the treatments are assigned by formal randomization. The reference standard and the unknowns, which may be urine extracts or pure steroids, are dissolved in an accurately measured amount (usually 1.0–1.2 ml.) of absolute ethanol and kept in 2.5 ml. glass vials with vinylite-lined screw caps. The vials are kept in a desiccator to minimize evaporation.

The unknowns are almost invariably assayed in two or more separate assays. In the first assay they are often tested at one dose level only. In subsequent assays they are tested at two dose levels with a fourfold difference between them. The dose levels are selected on the basis of the preliminary one-dose level assay so as to be bracketed by the standards. There is ample space for the simultaneous testing of several related unknowns, and direct comparisons can then be made between them, often with greatly increased efficiency (30).

The solutions are applied once daily for seven days (including Sunday) to the combs of the chicks by means of 0.01 ml. micropipets fitted with "bulbs" made from small-bore rubber tubing. Immediately before the application the feathers (down) near the comb are smoothed away from the comb with a slightly moistened finger. Routinely, the first application of androgen is made on the day of arrival in the laboratory (the day following hatching), but it may be deferred until the following day without obvious detriment to the assay.

The chicks are fed only cracked corn for the first two days in the laboratory and a mixture of cracked corn and mash in approximately equal proportions thereafter. Grit is also supplied. The delay in introduction of mash into the ration reduced the incidence

of diarrhea and "plugging" to an inconsiderable level.[4] On the evening of the seventh day of treatment the food is removed from the cages.

On the morning of the day after the last day of treatment the chicks are killed with ether, one group at a time, and weighed to the nearest 0.5 g. The comb is excised with a sharp No. 10 Bard-Parker scalpel, blotted on filter paper to remove blood, and weighed to the nearest 0.5 mg. on a Roller-Smith torsion balance. The groups of chicks are autopsied in the same sequence in which they have been subjected to daily treatment.

For convenience in the statistical computations the results are expressed (to two places) as

$$100 \times \log \frac{\text{comb weight in mg.}}{\text{body weight in dkg.}}$$

This metameter is obtained directly from the comb weight in mg. and the body weight in gm. by the use of either specially prepared tables or a nomogram.

The estimates of the androgenic potencies of the unknowns are computed by standard statistical procedures for parallel line assays (31). The potency of a urine extract is expressed in mg. of androsterone per 24 hours. The potency of a pure steroid is expressed as the % potency of androsterone. Both 68% and 95% confidence limits are computed for each potency estimate.

The standard errors, like the potency estimates, are first calculated in logarithmic units. When the potency estimates are converted into antilogarithms (R), the standard errors may also be converted to antilogarithms. Since addition of logarithms implies multiplication of antilogarithms, the $\pm$ symbols usually used for standard errors are changed to $\overset{\times}{\div}$ for the antilogarithms as shown in the following example:

	Estimate of potency		*Standard error*	*Approximate 68% confidence limits*
In logarithms	0.301	$\pm$	0.097	0.204–0.398
In antilogarithms	2.0 mg.	$\overset{\times}{\div}$	1.25	1.6 –2.5 mg.

In this example the standard error may also be cited as 25% of the estimate: (1.25–1.00) 100 = 25%. This type of computation produced the estimates of "expected minimum % $s.e._R$" shown in Table 1.

The best available estimate of the androgenic potency of an unknown is obtained by combining the estimates from two or more assays in the form of a weighted mean, as described by Bliss (31), both for homogeneous and heterogeneous replicate estimates. Similar calculations lead to the confidence limits of the weighted mean estimate.

EVALUATION OF THE CURRENT ASSAY PROCEDURE (SERIES V) IN PRACTICE

The results of 29 assays by the current assay procedure, representing our experience from December 1955 to November 1956, are summarized in Table 2. Routinely, three assays are run during each four-week period, and, with the exception of four special experimental assays (Nos. 146–149) and one (No. 133) in which the chick body weights were excessively variable, all the assays of Series V up to the end of November 1956 were included in the analysis.

In Table 2, the mean responses to the two dose levels of the reference standard, androsterone, are shown for each assay. The mean value of the control groups, chicks treated with ethanol only, is also shown for each of

[4] We are indebted to Dr. D. L. Coffin for this recommendation.

TABLE 2. STATISTICS FROM 29 ANDROGEN ASSAYS IN SERIES V

Assay	Mean response*			Residual variance (s^2)	Slope on standard (b_S)	λ_S
	Control	Standard androsterone				
		0.1 μg.	0.8 μg.			
129	48.2*	67.2	89.0	76	24.1	.362
130	46.4*	67.8	97.3	112	32.7	.324
131	—	74.8	95.2	124	22.6	.494
132	56.4*	64.8	96.0	99	34.5	.288
134	—	63.8	100.1	89	40.3	.234
135	—	68.2	99.0	86	34.1	.272
136	—	72.7	100.6	87	30.9	.302
137	52.3	75.9	105.0	127	32.2	.349
138	60.3	75.5	101.6	88	28.9	.324
139	52.4	77.8	103.3	95	28.2	.346
140	50.1	74.1	102.0	78	30.9	.286
141	54.8	75.8	103.3	72	30.5	.278
142	—	71.3	101.8	113	33.9	.314
143	56.5	74.2	99.7	97	28.2	.348
144	60.4*	76.8	105.8	142	32.2	.371
145	—	77.0	103.3	107	29.2	.355
150	—	72.0	102.5	118	33.8	.322
151	57.7	72.8	101.4	86	31.7	.293
152	49.8	67.2	89.4	91	24.6	.389
153	58.0	74.6	102.9	100	31.3	.320
154	52.3	73.6	99.6	118	28.8	.378
155	58.7	74.9	104.2	105	32.4	.316
156	57.2	77.2	100.2	91	25.5	.375
157	54.3*	76.2	99.6	125	25.9	.431
158	56.7*	75.6	97.8	90	24.6	.386
159	63.2*	81.1	107.2	89	28.9	.327
160	62.8*	78.8	101.5	79	25.2	.353
161	56.9	79.1	102.8	75	26.2	.331
162	54.9*	76.0	97.1	114	23.3	.459
Mean	55.5	73.7	100.3	99.1	29.5	.342
Standard deviation	4.5	4.3	4.2	18.0	4.1	.056

* The mean response is expressed as

$$100 \log \frac{\text{comb wt., mg.}}{\text{body wt., dkg.}}$$

and represents the mean of two groups of chicks (approx. 20) except where starred (*); there it is the mean of one group (approx. 10).

the 22 assays in which there were controls. In all assays with control groups the mean response to the dose level of 0.1 μg. of androsterone was significantly higher than the controls, at $P<.05$ in assay 132, at $P<.01$ in all other assays.

The residual variance per response (s^2) with over 200 degrees of freedom in each assay was, on the average, 99. The values for s^2, over the whole series, were heterogeneous ($P<.001$) by Bartlett's test.

The regression of the responses on the log dose of standard was highly significant ($P<.001$) in all 20 assays. The slopes (b_S) of the standard curves had an observed variance of 16.9. Since the predicted variances had a mean of 12.0, the ratio of the observed to the mean predicted variance, 1.40, is still not completely satisfactory ($.10>P>.05$) but represents a

considerably better agreement between these two estimates than was found in our earlier modifications of the method (29).

Precision of the Current Assay Procedure. In the 29 assays of Series V tabulated in Table 2, the mean value of $\lambda = s/b$, the most widely used index of precision of a bioassay, was 0.34, with a standard deviation of 0.056. A more realistic expression of the internal precision of an assay method is provided by the minimum expected standard error (s.e.) of a log potency estimate (M), in which the number of assay animals customarily used in assaying the potency of an unknown is also taken into consideration. In parallel line assay methods, of which the androgen assay is an example, the minimum value of this variable

$$(\text{s. e. }_M) = \frac{s}{b}\sqrt{\frac{1}{N_S} + \frac{1}{N_U}},$$

where N_S and N_U are the number of assay animals on standard and unknown, respectively. (The correction term for the precision of the slope (32) was not required in these assays since $g = s^2t^2/b^2\Sigma x^2$ was $\leq .05$ in all cases.) The relative convenience of our current assay procedure and the small amount of androgen required per chick make it possible to use large numbers of chicks and thus to counteract the low inherent precision of the method to a considerable extent. For the 29 assays of Series V, with 40 chicks on the standard and 20 chicks on the unknown, $\text{s.e.}_M = 0.093$, giving in nonlogarithmic terms 24% as the s.e._R, a quite acceptable value.

Another meaningful expression of the precision of a bioassay is based on the range of the confidence limits of a potency estimate. The 95% confidence limits of the log potency estimate $= M \pm t\ (\text{s.e.}_M)$. In our assays, $t = 1.96$, giving a value to this range of $(2)(1.96)(\text{s.e.}_M)$, or approximately 4 times the s.e._M. The approximate ratio of the upper confidence limit to the lower confidence limit in nonlogarithmic terms may be obtained by raising the antilog of s.e._M to the fourth power. For the tabulated assays of Series V, this ratio = 2.3. Formulas for the range of the confidence limits that incorporate g and the mean responses to standard and unknown have recently been published by Bliss (33).

Interassay Error. The several indices of precision that have just been presented were based on internal assay statistics and thus furnish estimates only of the expected or predicted error of the assay. The actual or observed error of an assay method, as distinguished from the expected error, can be ascertained only by analysis of the potency estimates obtained from repeated tests of the same unknown in different assays; it approaches the expected error, calculated from intra-assay statistics, as a minimum.

We have reported elsewhere in detail (29) an investigation of the observed error of the estimates of androgenic potency obtained with this bioassay method through its five modifications. The variance observed in 169 potency estimates of 75 unknowns (urine extracts) in the assays of Series

II was more than three times the variance predicted from intra-assay statistics, indicating the existence of a large and significant interassay error. At the time of our previous report (29), 28 urine extracts had been assayed in replicate in Series IV and V, and the variation was not significantly greater than predicted. However, more recent results in Series V, involving a large proportion of urine extracts from abnormal individuals, did reveal a significant ($P < .05$) discrepancy between the predicted and the observed variation. The total results to date from 125 potency estimates for 59 urine extracts assayed in Series IV and V gave an observed variance 33% greater than the prediction. In the same assay series the observed variance of 110 replicate potency estimates of 48 synthetic steroids was 82% greater than predicted. Although it would therefore appear that significant interassay error had persisted in Series IV and V, in which ethanol was the vehicle for application of androgen, it was still greatly reduced from that found in Series II, where ether was the vehicle.

DISCUSSION

The launching of a new biological assay method requires only the demonstration of a measurable dose-related biological response and preliminary information about details of procedure, specificity, and precision. The widespread, discriminating, and efficient utilization of the method, however, may be greatly promoted by the publication of more extensive experience with it, including various refinements in procedure and the pitfalls and limitations that have been revealed in time. This has been one of the principal objectives of the present paper.

In reporting a biological assay method it is now, more than formerly, the commendable practice to state the precision quantitatively, usually as the index, λ. In his reviews of androgen assay methods, Dorfman (10, 34) has cited the various values of λ associated with different methods. We suggest, however, that these values should be regarded as highly provisional in view of the well-known wide fluctuation of λ from assay to assay, and the limited amount of data on which these estimates were based. The estimates of λ presented in this paper should be more reliable, not because they are necessarily any more stable than those reported by others, but because they are supported by more extensive data.

The limitations of λ as an index of precision should also be recognized. It is helpful to supplement it with a statement of the minimum expected standard error, which, unlike λ, takes into account the number of assay animals and the spacing of dose levels. However, the ultimate test of the precision of an assay method is the observed reproducibility of estimates on the same unknown from one assay to another. The conclusion that our modifications in the androgen assay procedure led to a considerable improvement in precision was based on the evidence that the interassay error as well as the intra-assay error had been reduced substantially.

There are numerous possible applications of this assay method. We have

used it extensively for the assay of the total androgen content of extracts of acid-hydrolyzed urine. While methods involving column and paper chromatography for the chemical assay of androsterone and dehydroepiandrosterone may supplant the biological assay, it may still be of use in substantiating the identity of a fraction assumed to be a specific androgenic steroid. The method as we have developed it has an m.e.d. so low that amounts customarily used in paper chromatography can be measured. This low m.e.d. also makes it feasible to measure the low concentrations of androgen in tissues and biological fluids other than urine, such as spermatic (35) and adrenal venous blood and systemic blood, although these extensions of the method have not yet been adequately exploited. The method should prove useful in assessing the distribution of androgen in the edible tissues of domestic animals fed androgens for economic reasons. We have thus far assayed approximately 150 steroids for androgenic activity in a study of the relation between structure and activity and have been impressed by a rather remarkable consistency in the results. It is hoped that this study may contribute to the current search for anabolic steroids with minimal androgenic activity, and more fundamentally may help in the understanding of the biochemical basis for the differential effects of androgens on tissues.

The relative potencies of androgenic steroids may differ considerably from one biological assay method to another. Perhaps the most outstanding discrepancy between the methods involving, respectively, injections of androgens and direct applications to the comb is in the relative potencies of androsterone and testosterone. When injected intramuscularly in oil into the capon, testosterone is approximately 7 times as potent as androsterone (36), while in our method testosterone is only $42\%^{\times}_{\div}1.15$ as potent as androsterone. Discrepancies also exist between the capon, chick, and castrate rat assay methods with respect to the relative potencies of testosterone, testosterone propionate, and androsterone.

In our method the steroid is applied to the comb in a volatile solvent so that essentially a film of the steroid itself is deposited on the surface. The relative potencies of steroids in our method, therefore, are determined not only by differences in their intrinsic effects on cellular physiology but also by the differential rate of absorption and dissipation of the steroids and by local catabolic processes in the comb. However, in methods involving injections, a number of additional factors are important, such as the rate of transformation and conjugation in the liver, the rate of excretion in the urine and feces, and the competition of tissues other than the specific target organs for the steroid. Thus it would appear that a method in which the androgen is applied directly to the comb might be somewhat more indicative of intrinsic androgenic activity than methods involving injections.

It is impossible to say which of the laboratory assay methods is more closely correlated with the response in man because of inadequate clinical

data and the lack of a good feasible method in man. The discrepancies between assay methods should be viewed not as obstacles in the way of progress but as leads to the further elucidation of the comparative pharmacology of the steroids.

Acknowledgments

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A HISTOLOGICAL STUDY OF THE PANCREAS, LIVER AND KIDNEY BOTH DURING AND AFTER RECOVERY FROM ALLOXAN DIABETES[1]

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ABSTRACT

One hundred and eighty two hamsters were injected intravenously, via the ocular sinus, with a diabetogenic dose of alloxan. Their blood sugars were determined by an anthrone technique at intervals for a period of 30 weeks. Samples of the organs were taken under Nembutal anesthesia during various periods of the diabetic phase and from time to time after recovery. Tissues were fixed in: Bouin's, Flemming's and a saturated solution of picric acid in absolute alcohol and stained in: modified aldehyde fuchsin; gallocyanin chrome alum and periodic acid-Schiff with and without maltase digestion.

The islets of alloxan diabetic hamsters return to normal either by: recuperation of cells damaged or inhibited by the drug; by proliferation from small ducts; by transformation of acinar tissue. Intermittent and persistent diabetics recover through the latter mechanisms. In most cases, a return to physiological normal precedes that of histological normal by several weeks.

During hyperglycemia, the livers of persistent and intermittent diabetics show less stored glycogen but these return to normal concomitant with the fall in blood sugar. The R.N.A. content of the livers of severe diabetics is likewise reduced in hyperglycemia and also returns to normal with recovery.

Cast formation in the kidney as well as periportal fibrosis and focal necrosis of the liver occur among untreated animals, their incidence increasing with age. Alloxan may aggravate such conditions but is not necessarily the cause.

Diabetogenic doses of alloxan do not result in fatty metamorphosis of the liver but do: increase the size and number of lipid droplets situated at the periphery of hepatic cords; induce the release of lipids from the cords to be subsequently engulfed by Kupffer cells. Alloxan does not appear to be causally related to lipids found in or near fibrotic or necrotic areas.

ALTHOUGH several of the rodents have been shown to recover from alloxan diabetes (1–7), it was only recently (8) that the hamster was placed in this category.

Closely allied to this problem is the question of whether normal islet histology is attained at the same time. And further, if the typical islet picture is recreated, it would be of interest to know by what mechanism(s) this is accomplished. Here, the records are incomplete. Johnson (9) illustrated transformation of acinar tissue into islets. This, together with pro-

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liferation from ducts, reestablished the islands of guinea pigs. Allusions have been made by several investigators to a return to normal insular histology in the rabbit but methods were not described. As early as 1943, Dunn and McLetchie (10) suggested "healing" and "replacement" in the islands of Langerhans in alloxan diabetic rats. Later, Hughes (11, 12) demonstrated acinar transformation in that animal. A part of this paper will be devoted to these questions in the hamster.

From the earliest days of alloxan experimentation (13–16), conflicting opinions concerning pathological changes in other organs have appeared in the literature. The issue is too inconsistent to make feasible a review in this paper. Although Harris, *et al.* (17) found no permanent changes in either kidney or liver when a strictly diabetogenic dose is given, our preliminary studies seemed to indicate that something happened to these viscera, at least in certain cases. Therefore, we felt that this also should form a part of the report.

Another point which has received little attention is the histochemical study of the liver in either the diabetic or recovered states. In this investigation efforts were limited to glycogen storage capacity, nucleic acid and osmophilic lipids.

Last, but by no means unimportant, is the relation of age and sex to the effect of this drug upon recovery and visceral pathology in general.

METHODS

One hundred and eighty two hamsters, equally divided as to sex were injected intravenously (18) with the diabetogenic dose of alloxan (19). Their treatment and the classification of reaction subsequently used have been described elsewhere (8). At the time of sacrifice, samples of kidney, liver and pancreas were removed under Nembutal anesthesia, in most cases while the animal was still alive. Three fixatives were used: Bouin's; Flemming's; absolute alcohol, saturated with picric acid. All tissues were dehydrated and cleared through ethylene glycol and benzene, imbedded in Tissue Mat and sectioned at 4 to 7 micra.[2] The staining techniques used were: a modification of Gomori's aldehyde fuchsin (20), Lagerstedt's (21) gallocyanin-chrome alum; an adaptation of a periodic acid-Schiff Method (20). Lipids were demonstrated according to the procedure of Janes and McEnery (22).

OBSERVATIONS

Pancreas

Resistent group. Those animals who show no fluctuations in blood sugar after the I.V. injection of alloxan demonstrate no alterations in beta cells.

Transient diabetic recovering in first 2 weeks. When compared with normal islets it is easily seen that these fall within the normal range. The typical picture is usually attained within the first two weeks after blood sugar reaches normal limits.

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[2] The author wishes to thank Grace M. House and Robert Allen for invaluable assistance.

Transient diabetic, recovering in the third and fourth weeks. For the first 6 to 8 weeks after glucose values return to normal, few islets contain beta granulation in either the typical amount or distribution. Some show invasion of acinar tissue (Fig. 1) with evidence indicating transformation of the latter into islets (see also Fig. 4). Others have a very pale kind of granulation. A few appear normal.

On the other hand, animals recovering in either the third or fourth weeks but sacrificed 9 or more weeks after physiological recovery show buds of heavily-granulated beta cells associated with ducts (Fig. 2) and nests of similar cells squeezed in among the acini. Many normal-sized islets, irregular in outline but typically granulated are also seen throughout the pancreas.

Intermittent diabetic. The histological picture of the islets in such animals varies. If sacrificed in one of the hyperglycemic phases, all islets show many large areas of cavitation indicative of active degeneration. Occasionally, a few normal beta cells are seen scattered among these (Fig. 3).

If an animal is sacrificed 6 to 8 weeks after physiological recovery, there is ample evidence for both acinar transformation and duct proliferation (as in Figs. 1 and 5, respectively) but there is complete lack of normal beta granulation. This is common only after 9 or more weeks (Fig. 4).

Persistent diabetic. The histological picture in these cases also varies with the physiological state at the time of death. In an individual which had maintained a hyperglycemia over many weeks and was still in that condition when the sample was taken, the islets are completely devoid of granules. Occasionally, a small area of degeneration is observed. In other instances, where the diabetes had persisted for some weeks but the blood sugar values were gradually approaching normal, the situation is often similar to that seen in intermittents (Fig. 3).

It is interesting to notice the changes which occur in these animals with the passage of time after physiological recovery. At 3 weeks it is apparent that there are proliferations from the ducts (Fig. 5) and transformation of acinar tissue (as in Fig. 1) but it is equally clear that degeneration is also going on (Figs. 6 and 7). These same processes are continuing at 7 weeks but typical granulation is not yet present.

About the tenth week, small buds of heavily-granulated beta cells are visible along the ducts (as in Fig. 2) and nests of similar cells are often observed scattered among the acini. At the same time, some of the medium and large islets show cells at their periphery containing both zymogen and beta granules (as in Fig. 4). Generally, most of the islets of similar size are entirely normal as to granulation but may be irregular in outline.

Kidney

The most prominent type of kidney pathology encountered was cast formation (Figs. 8 and 9) associated with a variable degree of tubular and

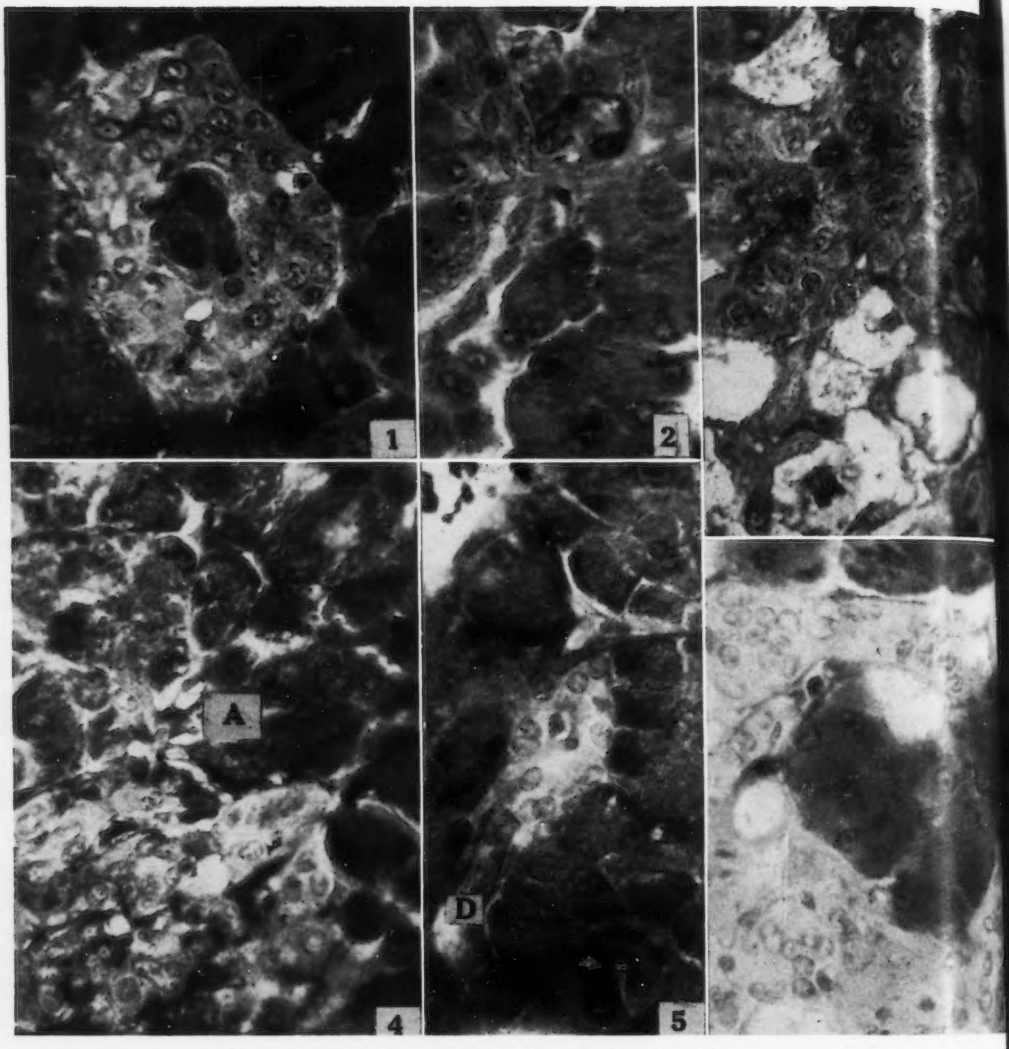

FIG. 1. Islet from transient diabetic recovering in the third week, sacrificed 8 weeks after recovery. Group of acinar cells in center of islet. Aldehyde fuchsin. ×550.

FIG. 2. Islet from transient diabetic recovering in the fourth week, sacrificed between 9 and 10 weeks after recovery. An inverted crescent of dark-staining beta cells is shown just at the edge of a duct. Aldehyde fuchsin. ×550.

FIG. 3. Section from islet of an intermittent diabetic sacrificed in its third hyperglycemic phase 78 days after injection. Shows cavitation with a few scattered, dark-staining beta cells. Aldehyde fuchsin. ×550.

FIG. 4. Section from an intermittent diabetic sacrificed 70 days after its third hyperglycemic phase. Most of the islet is normally-granulated. Just below "A," some acinar

capsular distension. In some cases, degenerative changes in both tubules and glomeruli were seen, but the vast majority of these were found only when cast formation was most pronounced.

Although admittedly, the most severe cases were discovered in hamsters which had been diabetic for long periods, it also happened that these animals were among the oldest in the group.

Since the incidence of this condition appears to be related to factors other than the intensity and duration of the hyperglycemic state, some of the findings which seem significant are presented at this point. Fourteen percent of the animals who were 8 to 9 weeks of age showed cast formation at the time of sacrifice and none of these were severe. Of this number, 75% were 6 to 11 months of age when the samples were taken. In the 6 months old group, 50% showed cast formation, generally of a moderate nature. Among the animals 9 months old or older at the time alloxan was injected, 70% showed this condition. Of these, over one third were severe. It is also worth mentioning that in the latter group (animals over 9 months of age) this type of pathology was found in several animals who were classified as alloxan resistant.

The sex of the animal apparently is not directly related to kidney pathology.

Liver

The report on the liver has been divided into two major parts: general and histochemical.

General. In this category, three aspects were considered: focal necrosis, periportal fibrosis (portal cirrhosis) and fatty metamorphosis.

This condition is rare in hamsters which were under 6 months of age at the time of injection. It has been found in about 9% of the cases. The most severe of these was seen in an animal which was about 6 months old when treated. However, at the time of sacrifice this specimen was over 14 months old. In animals over 6 months of age at the time of injection, the incidence of this condition was 30%.

As was the case of necrosis, this condition is rare in younger animals, appearing in only 4% of animals injected before they were 6 months of age. One of these instances was found in an animal injected at 8 to 9 weeks but was 9 to 10 months old when sacrificed. In hamsters over 6 months when

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cells are losing zymogen granules on one side and show dark, beta granules on the other. Aldehyde fuchsin. ×550.

FIG. 5. Section from pancreas of an animal diabetic for 12 weeks, sacrificed 3 weeks after recovery. This shows an oval, pale-staining cell cluster along a longitudinal section of a duct "D." Aldehyde fuchsin. ×550.

FIG. 6. Section from pancreas of an animal diabetic 9 weeks, sacrificed 3 weeks after recovery. This shows a deep indentation of darkly-stained acinar tissue into the pale islet tissue. A small focus of degeneration is seen. Aldehyde fuchsin. ×550.

treated, fibrosis was seen in 65% (Figs. 10 and 11). This condition has also been observed in older animals classified as alloxan resistant.

Using ordinary stains, no general change in fat content was found. Once more there is no relationship between the sex of the animal and liver pathology.

Histochemical. Three aspects are to be considered here: glycogen, nucleic acids and lipids.

During the diabetic phase either of the intermittent or persistent diabetic, glycogen storage is definitely below normal (compare Figs. 12 and 13). However, after physiological recovery glycogen is stored in the normal quantity (Fig. 14). The same distribution pattern is also found except in markedly pathological livers. For example, in cases of portal cirrhosis, the peripheral portions of the lobules are much depleted (Fig. 15).

The amount and distribution of lipids in the liver of untreated animals is highly variable and is, in part, dependent upon the hamster. In younger specimens, one finds thin strands of osmophilic particles occupying a narrow zone in the center of the hepatic cords. This pattern may appear in only small areas of widely scattered lobules (Fig. 16) or may be seen widespread throughout the section. In addition to these bands, medium and large spherules are often observed at the periphery of the cords. With age, the central strands may become less evident with an increase in size and numbers of peripheral droplets (Fig. 17) or these strands may enlarge to such an extent that they appear to occupy as much as two-thirds of the area of each cord. Often the peripheral osmophilic bodies bulge into the sinusoids.

There are 3 phenomena associated with the injection of alloxan. Of these, two are constant while the third appears to be incidental to other liver pathology. First, the size of the peripheral lipid droplets increases (Fig. 18). Many of these may clump together into even larger masses which often encroach upon the sinusoids. Second, some of this material is actually emitted by the hepatic cords only to be engulfed by Kupffer cells (Figs. 18 and 19). In a few instances there does appear to be a marked increase in the amount of the centrally-placed, fine granules (Fig. 18) but this is not consistent. The third change is seen only where degeneration and periportal fibrosis is observed. In such cases, cells loaded with osmophilic granules are seen scattered around necrotic areas (Fig. 20) while generally small but sometimes large (Fig. 21) droplets are seen in and around the walls of blood vessels.

With the equipment at hand, it was very difficult to determine variations in concentrations of D.N.A. or R.N.A. This situation is further complicated by the fact that there seems to be an increase in size and number of cytoplasmic chromidial substance (bodies) in older animals. However, there is a distinct impression that, in the persistent diabetic, sacrificed during hyperglycemia, there is less R.N.A. present. During the early weeks

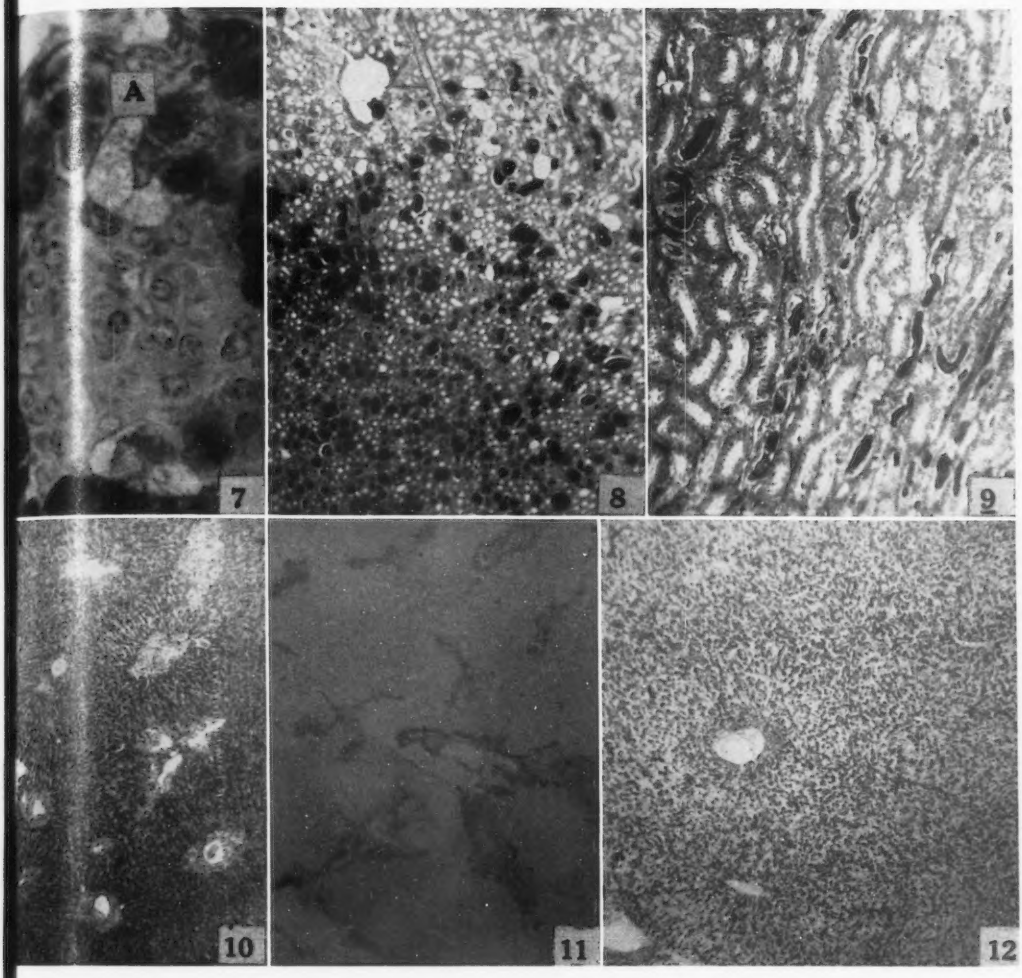

FIG. 7. Section from pancreas of animal diabetic 19 weeks, sacrificed 7 weeks after recovery showing degeneration of one group of acinar cells below "A", with the degranulation of others just to the left. Aldehyde Fuchsin. ×550.

FIG. 8. Section from kidney of an intermittent diabetic 12 weeks after its fourth hyperglycemic peak. Shows heavy, dark casts in most of the tubules of outer medulla (below) with tubular and capsular distension in the overlying cortex. Aldehyde fuchsin. ×100.

FIG. 9. Section from kidney of a transient diabetic recovering in the third week, sacrificed 11 weeks after injection showing a much milder case of cast formation with little distension. Aldehyde fuchsin. ×100.

FIG. 10. Section from liver of an intermittent diabetic sacrificed 12 weeks after its fourth hyperglycemic phase. Shows great increase in connective tissue around all blood vessels. Aldehyde fuchsin. ×30.

FIG. 11. Section from same liver from a maltase-digested Schiff preparation, all the irregular, dark areas representing periportal fibrosis. ×30.

FIG. 12. Liver of normal animal showing the typical glycogen distribution. Schiff. ×30.

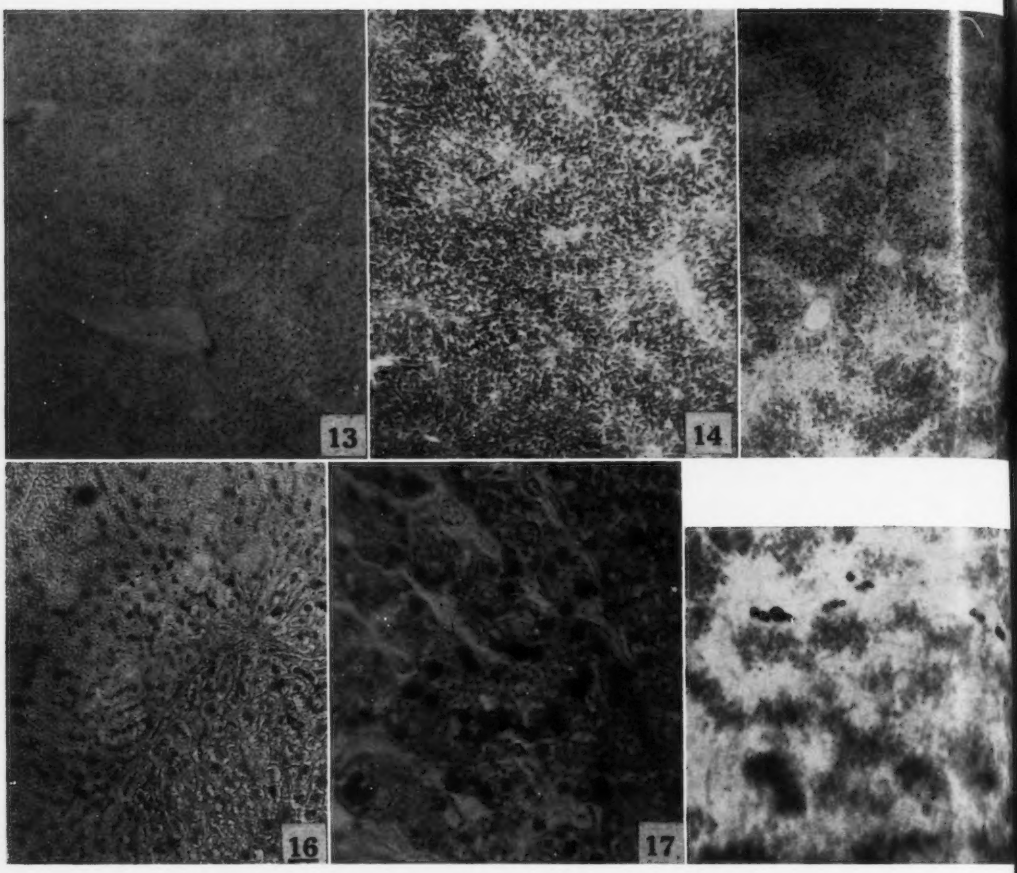

FIG. 13. Liver from an animal sacrificed in its third hyperglycemic phase showing much less glycogen than normal. Schiff. ×30.

FIG. 14. Liver from animal which was diabetic 7 weeks, sacrificed 5 weeks after recovery. Shows even more glycogen than the normal example (Fig. 12). Schiff. ×30.

FIG. 15. Liver from a transient diabetic sacrificed 12 weeks after recovery. This shows the glycogen pattern in a case of severe periportal fibrosis. Schiff. ×30.

FIG. 16. Liver from a young, normal animal showing a typical pattern of lipid distribution. Flemming's plus counterstain. ×100.

FIG. 17. Liver from an older, normal animal showing large and medium lipid droplets at periphery of liver cords. No central strands of fine droplets are seen. Flemming's plus counterstain. ×550.

FIG. 18. Liver from an intermittent diabetic sacrificed 10 weeks after its fourth hyperglycemic peak. This shows great increase in accumulation of fine droplets in center of cords, the clumping of larger drops at the periphery of the cords with an occasional droplet in a sinusoid. Flemming's plus counterstain. ×400.

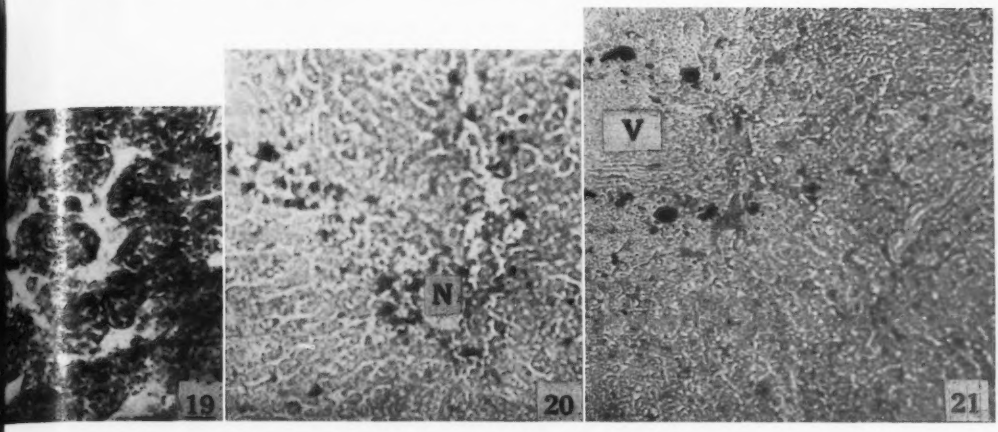

FIG. 19. Liver from an animal scarificed in its sixth week of hyperglycemia. Shows two osmophilic drops ingested by Kupffer cells of the sinusoids. Flemming's plus counterstain. ×550.

FIG. 20. Liver from an animal sacrificed in its ninth week of hyperglycemia showing some osmophilic substance around an area of necrosis "N." Flemming's plus counterstain. ×100.

FIG. 21. Liver from an intermittent diabetic sacrificed 12 weeks after its fourth hyperglycemic peak showing lipid droplets in and along wall of a blood vessel "V." Flemming's plus counterstain. ×100.

after recovery there actually may be a slightly greater than normal amount of this substance in the cytoplasm. In 8 to 10 weeks, the normal quantities are present.

There was little detectable difference in nucleic acid levels regardless of the blood sugar phase at the time of sacrifice among the intermittent diabetics.

All of the transient diabetics examined were sacrificed 3 weeks or more after recovery. No deviation from the normal nucleic acid content could be found.

DISCUSSION AND CONCLUSIONS

From the rapidity of the return to physiological and histological normal together with a lack of evidence supporting any other mechanism, it appears that transient diabetics recovering in the first two weeks do so by the simple recovery of beta cells which had been merely damaged or in some way inhibited by the drug. This possibility had been predicted on the basis of preliminary studies (House, Nace and Tassoni 25) during the first 24 hours when it was apparent that in some animals beta cells were degranulated but showed no degenerative changes.

It is possible that this same process plays a part in the reestablishment of normal islets in transient diabetics who recover in the third and fourth

weeks. However, there is ample evidence to show that acinar transformation and duct proliferation occur as well.

At this point, one might venture to say that there would be little reason to doubt the role of ducts in the replacement of islets since these are the precursors of all pancreatic tissue. However, the idea of the transformation of fully differentiated, zymogen-secreting cells into tissues equally as well specialized for the production of an entirely different substance, is distasteful to many, including some in our own group. The possibility of such transformation was suggested by Saguchi (23) as early as 1920 and certainly the evidence provided by Hughes (11 and 12) and Johnson (9) is quite clear. Although in the present study, we cannot exclude the possibility that some of the nests of beta cells seen scattered among the exocrine alveoli may arise from centro-acinar cells, the fact remains that in certain instances, cells containing both zymogen and beta granules have been observed. This would be indicative of transformation.

It can be said, without fear of equivocation, that both the intermittent and persistent diabetics recover through the medium of proliferation and transformation. However, a further word should be said about the appearance of such wide-spread degeneration in the islets of the former when sacrificed during an episode of hyperglycemia and how these are related to the fluctuations of blood sugar levels. It is believed that, sometime after beta cells were destroyed by the alloxan, regeneration begins, building up cells to a point where insulin secretion is able to reduce blood glucose to its normal value. However, perhaps due to insufficient numbers, many of these become exhausted and die. This permits another period of hyperglycemia but, due to the fact that at least some cells survive, this is not as severe as the preceding. This process is repeated perhaps several times until finally enough normal cells are present not only to maintain normal glucose values but to slow down the secretory activity to such an extent that the typical granulation picture reappears.

Some areas of degeneration are also seen in certain of the persistent diabetics, sacrificed while still hyperglycemic. Since this is more common in those cases which are approaching normal blood sugar levels, it is felt that this, too, is an exhaustion phenomenon on the part of cells recently regenerated.

It is also clear that at least part of the acinar tissue seen indenting or imbedded within islets degenerates (Figs. 6 and 7). Whether this occurs during the transformation process as well as at its conclusion could not be definitely established. There is some evidence that there is deterioration before definitive beta cells have been formed.

The fact that there is an extensive time lag between the return to physiological and histological normal has perhaps been misleading. On the basis of earlier observations, there had developed a feeling that perhaps some other adjustments were made to preclude the necessity for normally

granulated beta cells. It was not until a number of animals had been followed for many weeks after physiological recovery that the true situation became clear. Evidently, in all cases where proliferation and transformation are a major factor, about 8 weeks are required for the typical beta cell granulation to become reestablished. This is probably due to the fact that in the early stages of physiological recovery, secretion is discharged as fast as it is formed. Thus, whether one interprets the granules as insulin precursors or as stored hormone, the turn over is so rapid that there is no chance for accumulation. However, during this period, new cells are slowly being added until finally enough secretion is being produced so that the hormone synthesis is slowed down for all cells with the reappearance of granules as a consequence. Actually, during these early weeks, it is not unusual to find a scattering of areas of degeneration due, at least in part, to the exhaustion phenomenon described elsewhere.

Little additional comment will be presented at this time concerning pathology in the liver and kidney. It is obvious that the incidence of disease in both of these organs rises sharply with the age of the animal. However, since a comparable number of untreated animals of the same age ranges, maintained under similar environmental and dietary conditions were not available, one would hesitate to attribute all of these changes to the effects of the drug, particularly since Nace (24) has suggested that a certain amount of periportal fibrosis and focal necrosis occurs in the untreated hamster. It is probable that alloxan diabetes superimposed upon a preexisting fibrotic or necrotic liver aggravates both. The same could be said of the kidney in which cast formation had already begun.

It should not be surprising that the glycogen content of the liver returns to normal with physiological recovery. Although our study is not conclusive there is evidence that the histochemical picture of the liver becomes normal at about the time of physiological recovery and therefore precedes by several weeks the histological recuperation of the islets. Of perhaps greater interest is the fact that, at a time when the liver is most depleted of its glycogen, there is likewise less R.N.A. This would tend to indicate that there is a direct relationship between the latter and glycogenesis. As further support for this tenet is offered the increasing amounts of ribonucleic acid seen with the build up of glycogen reserves during and immediately after recovery.

For emphasis, it might be well to state that for a period of several weeks during which blood sugar is within the normal range and the liver shows normal glycogen content, the islets may show an almost complete lack of granulation.

At first glance the incidence of fat ingested by Kupffer cells after alloxan injection seems great. However, closer inspection shows that much of this is due to the increase in size and amount of peripheral lipid droplets. This is particularly marked in older animals. Observations presented here do

confirm the findings of Janes and McEnery (22) but is much less widespread in the hamster than in the rat.

Evidence has also been given to support the contention of Harris, *et al.* (17) that no permanent damage to the liver occurs when strictly diabetogenic doses of alloxan are used. Certainly there is no fatty metamorphosis, the slight infiltration of lipids in necrotic areas appearing to be incidental rather than causally related to the degeneration.

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ENZYMATIC ESTIMATION OF STEROIDS IN HUMAN URINE[1]

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ABSTRACT

The enzymatic microestimation of urinary steroid alcohols by means of α- and β-hydroxysteroid dehydrogenases is described. These highly purified enzymes catalyze selective and stereospecific oxidations of certain hydroxysteroids to ketosteroids. Diphosphopyridine nucleotide functions as hydrogen acceptor and the assays are based on the spectrophotometric measurement of the reduced pyridine nucleotide. Methods are described for the preparation of extracts of human urine suitable for enzymatic assay. The reliability of the procedures has been established by a variety of criteria including the recovery of physiological amounts of steroids added to urine. Values for the excretion of α- and β-hydroxysteroids have been determined in normal adult subjects of both sexes and in several patients with abnormalities of the endocrine system. The estimation of individual urinary steroids separated by paper chromatography is demonstrated.

THE estimation of urinary steroids in man has been of interest for approximately 20 years since the introduction by Zimmermann (1) of a simple colorimetric test for 17-ketosteroids (17-KS.), the determination of which has been widely practiced and clinically useful. No other analytical method for urinary steroids has achieved the popularity and utility of the Zimmermann reaction or one of its numerous modifications (1, 2, 3). Attempts to appraise more fully the urinary excretion of steroids have led to the development of a number of colorimetric methods designed to be specific for other groups of steroids, such as the metabolites of adrenal cortical hormones, estrogens and progesterone (4). The aims of all analytical techniques have been to combine simplicity of operation with accuracy and specificity, objectives which have not been entirely compatible. The principal methods for the estimation of steroids have been critically discussed recently (4, 5). The chief drawbacks in the presently available chemical methods are their lack of specificity for steroids in general or even specific groups of steroids. Thus, from an extensive study of the specificity of the Zimmermann reaction, Wilson (6), as well as others, have concluded that

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[2] Scholar in Cancer Research of the American Cancer Society.

many compounds other than 17-KS.[3] react to a significant extent. Moreover, the relative chromogenicities of various 17-KS. and of other ketosteroids are highly variable and depend critically upon the conditions under which the reaction is carried out.

The need for a convenient method for measuring steroid metabolites which is steroid specific and at the same time all-inclusive, has been pointed out (3). Interest centers on the measurement not only of total steroid excretion but also on the accurate estimation of groups or of individual urinary steroids. A survey of the known neutral urinary steroids reveals that a large proportion of these C_{19} and C_{21} compounds carry either 3α- or 3β-hydroxyl groups, whereas only a minority are 17-KS. (Table 1). The list of

TABLE 1. CLASSIFICATION OF NEUTRAL URINARY STEROIDS ACCORDING TO FUNCTIONAL GROUPS (7)

Group	No. of compounds
Total	53
3α-Hydroxysteroids	26
3β- and Δ^5-3β-Hydroxysteroids	17
17-Ketosteroids	14
Saturated 3-Ketosteroids	7
Δ^4-3-Ketosteroids	3

neutral urinary steroids given by Dorfman and Ungar (7) in 1953, which is now somewhat incomplete, demonstrates this fact clearly. Thus, 43 of the 53 well-defined urinary steroids listed bear either 3α- or 3β-hydroxyl groups. Fourteen of the 53 compounds are 17-KS., and 9 of the latter 17-KS. also bear hydroxyl groups at position 3 (Table 1). Hence, a procedure for estimating steroid alcohols is applicable to the majority of steroid metabolites. An ingenious method of using the alcoholic group of steroids in the estimation of urinary metabolites has been devised by Engel and Baggett (8), who have also critically reviewed the procedures of others for performing such estimations.

Within the past few years, two purified bacterial hydroxysteroid dehydrogenases have been prepared which carry out stereospecific interconversions of certain steroid alcohols and ketones (9, 10). These enzymes have been shown to be suitable for the microestimation of certain steroid alcohols and steroid ketones (10, 11). Although tedious to prepare, the enzymes are ideal reagents for the estimation of steroids since they offer advantages of extreme specificity and sensitivity. The specificity of the two purified α- and β-hydroxysteroid dehydrogenases is such as to permit the

[3] The following abbreviations are used: DPN^+ = oxidized diphosphopyridine nucleotide; DPNH = reduced diphosphopyridine nucleotide; α enzyme = 3α-hydroxysteroid dehydrogenase; β enzyme = 3β- and 17β-hydroxysteroid dehydrogenase; THE = tetrahydrocortisone (pregnane-3α,17α,21-triol-11,20-dione); α-H.S. = 3α-hydroxysteroids; β-H.S. = 3β-(and 17β-)hydroxysteroids; 17-KS. = 17-ketosteroids.

measurement of 3α- and 3β-hydroxysteroids, as well as 3- and 17-ketosteroids (11). The only compounds in Table 1 which are not measurable by the enzymatic methods are the three Δ^4-3-ketosteroids.

Although the measurement of various enzyme activities in blood and urine has been a useful diagnostic tool for some time (12) recognition of the utility of enzymatic methods for measuring biological constituents has been much more limited. Within the past few years Bonnichsen and Theorell (13) in Sweden and Bücher and coworkers (14) in Germany have introduced an enzymatic method for the estimation of human blood alcohol levels for medico-legal purposes. Their methods and those of the present paper depend upon the utilization of enzymes requiring diphosphopyridine nucleotide (DPN^+) as a coenzyme. Hydroxysteroid dehydrogenases carry out the oxidation of hydroxysteroids to ketosteroids with the concomitant reduction of the DPN^+ to DPNH, according to the following equation:

$$\begin{matrix} \diagdown \\ \diagup \end{matrix}\!\!\text{C—OH} + DPN^+ \rightleftharpoons \begin{matrix} \diagdown \\ \diagup \end{matrix}\!\!\text{C=O} + DPNH + H^+$$

where the partial formulae refer to steroid alcohol and ketone. DPNH is characterized by an absorption peak at 340 mμ which has a relatively high molar extinction coefficient ($\epsilon = 6{,}220$) in the near ultraviolet region where the oxidized coenzyme (DPN^+) is optically transparent. The amount of DPNH formed thus is in stoichiometric relation to the amount of steroid alcohol oxidized. The value of such optical methods for the measurement of enzymes and substrates was first recognized by Warburg who determined the structure of DPN^+ and demonstrated the changes in its absorption spectrum upon reduction (15).

The preparation of purified α- and β-hydroxysteroid dehydrogenases, their specificity, and the equilibria catalyzed by these enzymes have been described (9–11). By taking advantage of the specificity of the hydroxysteroid dehydrogenases and by displacing equilibrium conditions of reactions such as to favor either complete oxidation or reduction of steroids it has been possible to devise convenient and sensitive enzymatic micromethods for the estimation of urinary steroids. A brief account of the suitability of enzymatic assay methods for the estimation of total urinary 3α-hydroxy-, 3β-hydroxy-, 3-keto- and 17-ketosteroids has been given together with preliminary values for normal men and women (16). It is the purpose of the present communication to describe our experience with the enzymatic estimation of urinary steroids in various endocrine abnormalities and to give more information on normal subjects. Since the measurement of groups of steroids is a composite figure, which is necessarily of only limited usefulness, some consideration is also given to the estimation of individual urinary steroids when these compounds have been separated by paper chromatography. The preparation of urine extracts suitable for enzymatic

analysis, the hydrolysis of steroid conjugates and the recovery of steroids added to urine have also been studied.

MATERIALS AND METHODS

Materials and Preparations. DPN^+ of high purity was purchased from Pabst Laboratories, Milwaukee, Wisconsin. A solution containing approximately 5 μmoles per ml. was prepared by dissolving 41 mg. DPN^+ in 10 ml. water and neutralizing with $NaHCO_3$. This solution should be kept cold and stored frozen. Hydrazine hydrate (100%) was obtained from Fairmount Chemical Company, 136 Liberty Street, New York. 5.0 ml. of the hydrazine hydrate were dissolved in H_2O, 1.5 ml. 2N H_2SO_4 were added carefully and the volume made up to 100 ml. giving a 1 M solution of approximately pH 9.5. It is more convenient to use the liquid hydrazine hydrate (100%) than crystalline hydrazine sulfate as previously recommended (11, 16) Reagent grade methanol was redistilled. Water was distilled in an all glass still. Reagent grade methylene dichloride was distilled from K_2CO_3. Phillips Petroleum Company n-hexane was purified by treatment with several changes of conc. H_2SO_4, followed by alkaline $KMnO_4$, dried over sodium and distilled through a fractionating column. Amberlite MB 1 was obtained from Rohm and Haas Company, Philadelphia. The resin was washed in large columns with distilled water and then CH_3OH, until the CH_3OH washings from the column showed negligible light absorption at 340 mμ. Purified beef liver glucuronidase (Ketodase) was supplied by Warner-Chilcott Laboratories, New York and was stated by the manufacturer to assay 5000 units per ml. α and β enzymes were prepared according to the method of Marcus and Talalay (9), as modified by Hurlock and Talalay (11). The enzyme preparations had specific activities of at least 30,000 to 50,000 units per mg. of protein. The α enzyme contained negligible amounts (less than 0.1%) of β enzyme, whereas β enzyme preparations were usually contaminated to the extent of 1 to 2% with α enzyme activity.

All evaporations were carried out in a rotating evaporator *in vacuo* below 45°. Small volumes were evaporated in a stream of N_2. All optical measurements were conducted in a Beckman DU Spectrophotometer in cuvettes of 1 cm. light path at approximately 25° and at 340 mμ. Cuvette windows were either pyrex or silica. The pyrex cuvettes are less expensive and completely satisfactory.

Methods. The assays were conducted essentially as described (11, 16). The assay systems contained 1.0 ml. 0.1*M* sodium pyrophosphate buffer (pH 9.5), 0.1 ml. DPN^+ solution (5 μmoles per ml.), 1.0 ml. 1*M* hydrazine, 0.01 to 0.1 ml. of urine extract in methanol. Methanol was added so that the final volume of CH_3OH per cuvette was usually 0.1 ml. The system was made up with H_2O to give a final volume of 3.0 ml. The control cell contained all ingredients including 0.1 ml. CH_3OH in place of the urine extract. Each extract was assayed at three concentrations, usually 0.025, 0.050, and 0.075 ml. Initial readings of the optical density at 340 mμ were taken, and then 500 to 1000 units of α and β enzymes in 0.01 to 0.02 ml. each were successively added. Readings of the optical density were taken at intervals until the reaction was complete with α enzyme, before adding β enzyme. The measurements are based on optical density changes at 340 mμ at equilibrium, first utilizing α enzyme and then β enzyme. This order is important because of the contamination of β enzyme by α enzyme. Assuming the molar extinction coefficient for DPNH is 6,220 at 340 mμ, the calculation of the amount of DPNH formed or steroid oxidized in micromoles in the 3.0 ml. system involves division of the observed change in optical density by 2.07.

Extracts of urine suitable for enzymatic analysis were prepared as follows. A 25 ml. aliquot of a 24-hour urine collection was measured into a glass-stoppered Maizel-Gerson vessel (Wilkens-Anderson Co., Chicago). The urine was acidified to pH 4.5 with

dilute H_2SO_4. 1.25 ml. 1.0 *M* sodium acetatebuffer pH 4.5 and 5.0 ml. β-glucuronidase (25,000 units) were added and the mixture incubated at 30° for 24 hours. Following extraction with three 15 ml. portions of CH_2Cl_2 (centrifuged if necessary to break emulsions), the aqueous residue was acidified to pH 1 with dilute H_2SO_4 and incubated at room temperature for 24 hours longer. The residue was reextracted with three 15 ml. portions of CH_2Cl_2 (centrifuged if necessary) and the combined solvent extracts evaporated to dryness. The residue was dissolved in 5 ml. CH_3OH and passed slowly (10 minutes per passage) once or twice over a 70×10 mm. column of Amberlite MB 1 in a 60 ml. capacity globe-shaped separatory funnel with long stem. The column was finally washed with a total of 25 ml. of CH_3OH. The combined column eluates were evaporated to dryness and dissolved in 15 ml. aqueous methanol (70 CH_3OH:30 H_2O by volume) and extracted three times with 5 ml. portions of n-hexane. Each hexane extract was back-washed with aqueous CH_3OH. The aqueous methanol phases were combined and evaporated to dryness. The residue was redissolved in CH_3OH, transferred to a small glass-stoppered vial, evaporated to dryness in a stream of N_2 and redissolved in exactly 0.5 ml. CH_3OH. Aliquots of this extract amounting to 0.025 to 0.10 ml. were used in the enzymatic assays.

RESULTS

Preparation of Urine Extracts and Steroid Recoveries. The quantitative extraction of steroids from urine in a form suitable for enzymatic analysis was investigated. It is desirable that there be no measurable turbidity at 340 mμ upon mixing all the components of the reaction system. Since the assays are performed in an almost completely aqueous medium, turbidities may result from two causes: the presence of water-insoluble lipids in the extract, or the precipitation of the enzyme proteins by the urine extract. The water-insoluble lipids can be effectively eliminated by partitioning the urine extract between aqueous methanol and hexane. Precipitation of the enzymes by the urine extract was not observed with enzyme preparations having specific activities of 30,000 units per mg. of protein or higher, because of the relatively small amounts of protein which were added to the cuvettes. Urine extracts of exceptionally low optical density (at 340 mμ) were obtained by passing the extracts slowly over short columns of Amberlite MB 1 (a strong acid-base mixed bed ion exchange resin) which effectively replaced the widely used wash of urine extracts with base and quantitatively removed phenolic estrogens.

In order to investigate the possible loss of steroids during individual steps of the purification of the extract, the content of α- and β-H.S. was measured after various treatments of the extracts. Aliquots of an urine extract were subjected to the following procedures: 1. Passage over an Amberlite column in CH_3OH; 2. Washing of the CH_2Cl_2 extract with 0.1N NaOH, followed by passage over Amberlite in CH_3OH; 3. Washing of the CH_2Cl_2 extract with 0.1N NaOH, followed by partition of the extract in aqueous methanol (70 CH_3OH:30 H_2O) against hexane. 4. Passage over Amberlite in CH_3OH, followed by partition against hexane. Following these procedures aliquots of each extract were assayed with α and β enzymes. All of the procedures resulted in negligible ($\pm 5\%$) losses of neutral

steroids. However, the ion-exchange resin treatment, together with hexane partition gave the clearest extracts with the least optical density at 340 mμ. Urine extracts prepared in this manner have been repeatedly examined for suitability in enzymatic analysis. Such extracts do not cause precipitation of enzyme protein. In the absence of enzyme, these extracts do not reduce DPN^+, and do not reoxidize DPNH under the conditions used in the assays.

In order to evaluate the possible loss of steroids in the entire procedure of preparing the extract, some of the most abundant urinary steroids of widely differing polarities (androsterone and THE) were added in physio-

TABLE 2. RECOVERIES OF ANDROSTERONE AND THE ADDED TO URINE*

Material from which extract was prepared	Micromoles 3α-hydroxysteroid per 0.5 ml. extract	Calculated	Discrepancy %
Urine	0.779	—	
Urine + Androsterone	1.33	1.43	−7.0
Urine + THE	1.26	1.36	−7.4
Androsterone Solution	0.654	—	
THE Solution	0.585	—	

* To a 25 ml. aliquot of normal male urine 1.0 ml. of a solution of androsterone in CH_3OH was added. To a second 25 ml. aliquot 1.0 ml. of a solution of THE in CH_3OH was added. 1.0 ml. of CH_3OH was added to a third 25 ml. aliquot of the urine. All three aliquots were then separately carried through the entire procedure of preparing a urine extract, including hydrolysis with glucuronidase and at pH 1, ion-exchange treatment, and hexane partition. The final volume of the CH_3OH extract was 0.50 ml. in each case. The steroid content of the androsterone and THE solutions were estimated separately by evaporating 1.0 ml. aliquots of these solutions in a stream of nitrogen and redissolving in 0.50 ml. CH_3OH. All the five 0.50 ml. CH_3OH extracts were then assayed enzymatically with α enzyme using 0.025, 0.050 and 0.075 ml. aliquots. The values given are the means of these determinations and are expressed as total μmoles 3α-H.S. in the entire extract.

logical amounts to an aliquot of urine which was then subjected to the complete procedure of preparation of an extract, including hydrolysis with glucuronidase and at pH 1, ion-exchange resin treatment, and hexane partition. The recovery of androsterone was 93% and that of THE was 92.6% when assayed with α enzyme (Table 2).

Hydrolysis of Steroid Conjugates. The availability of rapid enzymatic methods for the measurement of free steroid alcohols provided an opportunity for a study of the effectiveness of various hydrolytic procedures in liberating these alcohols from their urinary conjugates. A limited investigation of the hydrolysis of conjugates of α-H.S. was undertaken. A 100 ml. aliquot of urine was acidified to pH 4.5 with 2N HCl, and extracted at once with three 100 ml. portions of CH_2Cl_2. The combined CH_2Cl_2 extracts were dried over anhydrous Na_2SO_4, evaporated to dryness and dissolved in 5.0 ml. CH_3OH. Suitable aliquots of this solution were assayed with α enzyme. The results are graphically presented in Figure 1 (A). A second 100 ml. aliquot of the same urine was acidified to pH 4.5 with 2N HCl, 5.0 ml. 1.0*M* sodium acetate buffer pH 4.5 and 20 ml. (100,000 units) β-glucuroni-

dase were added and the mixture incubated 15 hours at 35°. The glucuronidase-hydrolyzed material was extracted as described and a CH_3OH extract assayed with α enzyme (Fig. 1, B), the result being corrected for the amount of 3α-hydroxysteroid found in unhydrolyzed urine (A). At this time, 10 ml. (50,000 units) more β-glucuronidase were added to the already extracted urine and incubation continued for 18 hours longer at 35°. The extract obtained from this material was designated as C, Figure 1. The residual urine from both glucuronidase hydrolyses was then acidified with H_2SO_4 to pH 1 and maintained at about 25° for 18 hours. Upon re-extraction and assay with α enzyme, the material was designated D, Figure 1. Finally, the pH 1 urine residue was placed on a steam bath at 100° for 1 hour, and another extract prepared and assayed, Figure 1, E.

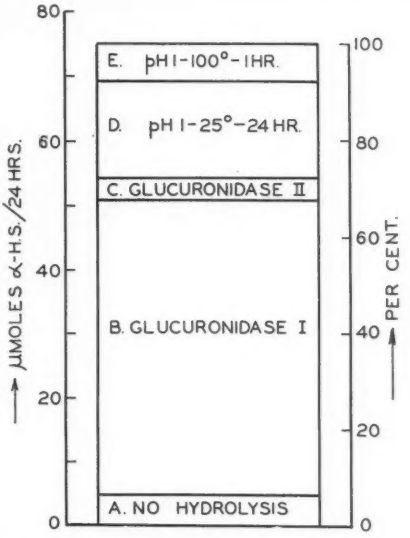

FIG. 1. Amounts of 3α-H.S. liberated by the action of various hydrolytic procedures on normal male urine. The values are expressed as micromoles per 1000 ml. urine and as percentages of the total liberated by the combined procedures. Details of the steps are given in the text.

The amounts of α-H.S. liberated by each of these treatments are expressed in Figure 1 as micromoles per liter of urine and as percentages of the total amount of α-H.S. liberated by all the treatments combined. Only 6.5% of the total α-H.S. are unconjugated. Nearly 66% of the total conjugates are liberated by glucuronidase hydrolysis and another 20% by hydrolysis at pH 1 at room temperature. The latter procedure presumably splits the acid-labile sulfates. The final treatment at 100° and pH 1 for one hour only freed 7.8% of the total. In separate experiments in which the urine was treated directly at pH 1 and 100° for one hour, only 60% of the maximum amount of α-H.S. liberated by the stepwise procedure was found. The hydrolysis of β-H.S. was not studied. The glucuronidase preparations used were free from detectable amounts of α- and β-H.S.

Linearity of Assay with Respect to Volume of Urine Extract. Although each urine extract has been routinely assayed at two or three concentrations, the assay procedure was checked for linearity over a wide range of concentrations. An extract of male urine was prepared as described except that 1.0 ml. extract was equivalent to 15.0 ml. urine. Aliquots of this extract were assayed with α enzyme and then β enzyme in a 3 ml. system utilizing 0.025, 0.050, 0.075, 0.20, 0.25 and 0.30 ml. of the extract. Over

this 12-fold range of concentrations, individual values did not deviate significantly from the mean, as may be seen from Figure 2. It may be concluded that the reaction equilibrium is completely displaced in favor of oxidation of the steroids, and that accurate measurements of these compounds are therefore being made. Figure 2 shows that this urine extract, which is representative of others, gave an initial optical density at 340 mμ of 0.039 for 0.2 ml. extract per 3.0 ml. system. Upon the addition of α enzyme the optical density rose to 0.470 (change in optical density = 0.431) and upon further addition of β enzyme it rose to 0.535 (total change in optical density = 0.496), so that the blank amounted to approximately 7 to 8% of the total optical densities measured. The optical density of the urine extract varied linearly with the amount of extract added to the cuvette (Figure 2).

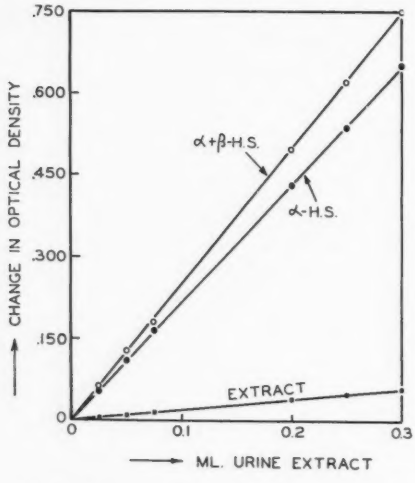

FIG. 2. Linearity of assays for α- and β-H.S. with amounts of urine extract. The extract was prepared as described under Methods from the urine of a 32-year-old man (total 24 hour volume = 1050 ml.). A 300 ml. aliquot of the urine was processed to give a final extract of 20 ml. in CH_3OH (i.e. 1.0 ml. extract = 15 ml. urine). The assays are expressed as increases in optical density (340 mμ) at equilibrium produced by α and then β enzyme reactions in a 3.0 ml. system. The lowest line shows the optical density (340 mμ) of varying volumes of the urine extract.

Hydroxysteroid Excretion in Normal Subjects. The α- and β-H.S. were determined in 25 ml. aliquot volumes of 24-hour collections of urine in 13 men (age range 20 to 47 years) and 11 women (age range 17 to 37 years) all believed to be in good health. The results are shown in Table 3, expressed as concentrations in μmoles per liter and as total daily amounts in μmoles per 24 hours. In this series of normal subjects which is about twice as large as that previously reported (16), and includes the former determinations, there is no difference in the mean values of 24-hour excretions in men and women. The values per liter of urine do not show a larger standard deviation than those expressed as 24-hour collections. The individual values of α- and β-H.S. excreted per 24 hours are shown in Figure 3. A correlation of the magnitudes of the α- and β-H.S. is apparent. The Pearson product correlation coefficient (17) for the values of men is $r = 0.468$ (not significant at the 0.05 level) and for women $r = 0.744$ (significant at the 0.01 level).

TABLE 3. URINARY EXCRETION OF α- AND β-HYDROXYSTEROIDS IN NORMAL ADULT SUBJECTS

Subjects	No.	α-Hydroxysteroids		β-Hydroxysteroids	
		μmoles/24 hrs.	μmoles/L	μ moles/24 hrs.	μmoles/L
Men (20 to 47 years old)	13	43.7 ± 13.0 (29.1 to 70.7)	41.8 ± 14.1 (18.7 to 66.7)	7.52 ± 2.71 (3.16 to 13.7)	6.90 ± 2.75 (3.29 to 13.2)
Women (17 to 37 years old)	11	42.4 ± 18.5 (16.4 to 74.2)	44.1 ± 16.1 (26 to 78)	6.07 ± 2.65 (2.61 to 11.6)	6.44 ± 2.87 (2.90 to 14.6)

The values are given as means together with standard deviations.
The values in parentheses indicate the lowest and highest values in each group.

The ratios of α-H.S. to β-H.S. for men is 5.8 and for women 7.0 calculated on a basis of the mean values. The molecular weights of the common urinary steroids vary from 288 (dehydroepiandrosterone) to 368 (tetrahydrocortisol), and hence the micromolar values can be converted to approximate values in milligrams by dividing by 3.

Hydroxysteroid Excretion in Various Endocrine Diseases. Steroid excretion in a series of patients with diseases of the endocrine system or surgical ablations of certain endocrine glands has been investigated. Results of these measurements of urinary steroids together with clinical details are

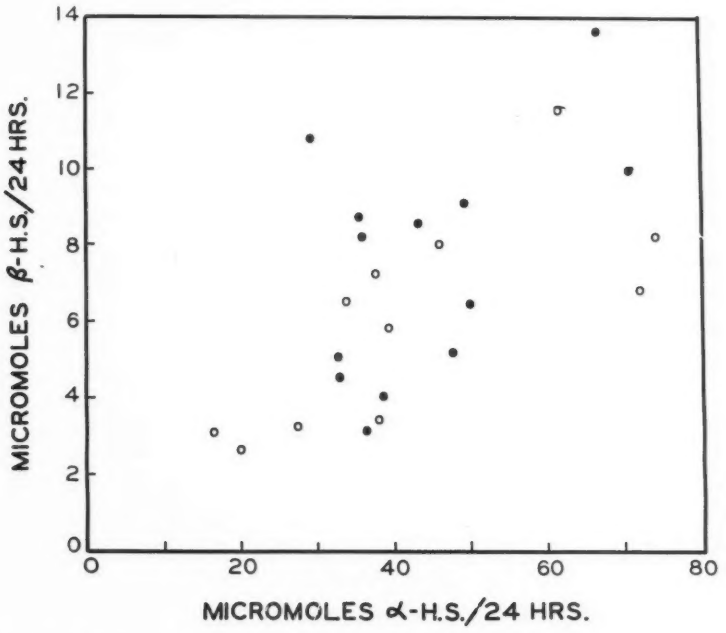

FIG. 3. Relation between urinary excretion of β- and α-H.S. (micromoles per 24 hours) in normal subjects. ○ Women; ● Men.

given in Table 4. Patient No. 1 with a clinical diagnosis of the adrenogenital syndrome had definite elevations of 17-ketosteroids, both by enzymatic assay and Zimmermann reaction. The β-H.S. were greatly elevated (about 5 times normal), whereas the α-H.S. were at the upper limits of normal. Upon treatment with cortisone acetate (25 mg. by mouth daily), this patient showed a lowering of her α-H.S. excretion to approximately one half, whereas the β-H.S. were more markedly reduced to approximately 7% of their pre-treatment values. The assays of the hydroxysteroid fractions in the urine of this patient before and after treatment are shown in Figure 4, which emphasizes the dramatic changes in the β-H.S. fraction.

A young woman with adrenal hyperplasia (Patient No. 2) showed less pronounced clinical signs of adrenal disturbance, characterized by hirsutism, obesity, and irregular menses. She had a mild elevation of α-H.S., whereas the β-H.S. were normal. Patient No. 3 (Table 4), a young man, with clinical hypopituitarism, excreted normal amounts of β-H.S., relatively low amounts of α-H.S., and decreased 17-KS. as measured by the enzymatic method. Excretion of α- and β-H.S. in a woman (Patient No. 4,

TABLE 4. URINARY STEROID EXCRETION IN PATIENTS WITH VARIOUS ENDOCRINE ABNORMALITIES

Patient No.	Age and sex	Description of patient	μmoles per 24 hours		
			α-H.S.	β-H.S.	17-KS.
1	46 F	Adrenogenital syndrome. Untreated	78.2	35.5	32.8
		25 mg. Cortisone acetate p.o. daily for 6 months	39.2	2.40	—
2	17 F	Virilizing adrenal hyperplasia	94.5	10.3	—
3	18 M	Hypopituitarism	18.2	9.00	2.54
4	44 F	Metastatic Carcinoma of Breast			
		6-9-56 5 yrs. after radical mastectomy and bilat. oophorectomy	28.3	4.70	—
		6-17-56 after bilateral adrenalectomy. On 50 mg. cortisone acetate p.o. daily	17.0	1.24	—
5	15½ F	Turner's Syndrome	24.2	3.62	—

Brief clinical histories of these patients follow:

(1) Alopecia; facial and trunk hirsutism; regular and normal menstruation; high 17-ketosteroids as determined by the Zimmermann method (30–40 mg. per 24 hours at various times).

(2) Obesity; irregular and scant menses; facial hirsutism; adrenal hyperplasia found on exploration.

(3) Short stature (4 ft. 10″ tall), high pitched voice; lack of axillary and pubic hair; small testes; infantile penis; anemia (Hgb. 11.5 gm.); old Legg-Perthes disease.

(4) Left radical mastectomy 1951 for carcinoma of breast. Two weeks later bilateral oophorectomy and X-ray therapy to scar. 1955 cobalt bomb therapy to chest. Developed pleural effusion. Bilateral adrenalectomy 6-11-56. On 50 mg. cortisone acetate p.o. daily since 6-17-56.

(5) Short stature (4 ft. 2 in.), cubitus valgus, no axillary and only very sparse pubic hair, no menses. Urinary estrogen excretion very low and follicle-stimulating gonadotrophin very high. Presumptive diagnosis Turner's syndrome (ovarian dysgenesis).

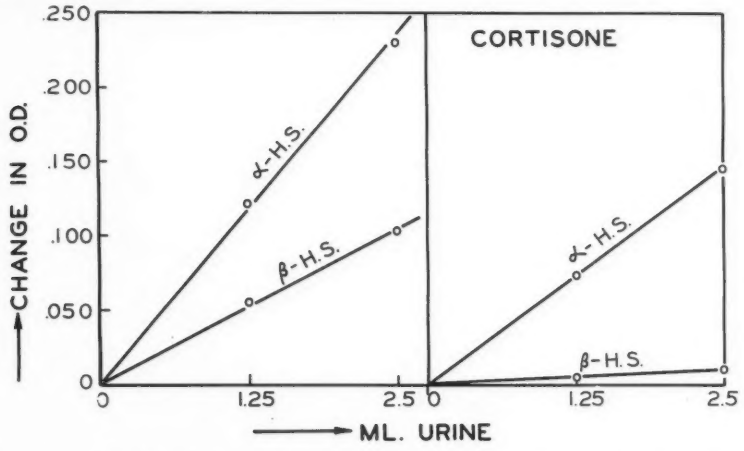

FIG. 4. α- and β-hydroxysteroid excretion in urine of a patient with adrenal cortical hyperplasia. On the left, control period (urine volume = 1700 ml./24 hr.); on the right, after treatment with cortisone acetate, 25 mg. daily (urine volume = 1380 ml./24 hr.). The change in optical density (340 mμ) produced by α and β enzymes is shown as a function of urine volume equivalent used for the assays.

Table 4) with metastatic carcinoma of the breast, 5 years after radical mastectomy and bilateral oophorectomy was relatively normal. One week after bilateral adrenalectomy, with maintenance on 50 mg. of cortisone acetate daily, the α-H.S. were at the lower limit of normal, whereas the β-H.S. were found to be one third of the lowest normal value, both α- and β-H.S. having declined after the operation. In a case of Turner's syndrome (Patient No. 5) in a $15\frac{1}{2}$ year old girl having both low estrogen and very high gonadotrophin excretion in the urine, the α-H.S. excretion was nor-

TABLE 5. URINARY STEROID EXCRETION IN A WOMAN WITH CARCINOMA OF BREAST AND AN OVARIAN TUMOR (THECOMA)*

Date	Procedure	μmoles per 24 hours		
		3α-H.S.	3β-H.S.	17-KS.
2-10-56	—	31.4	6.55	6.55
2-21-56	Bilateral Oophorectomy	—	—	—
2-26-56	—	5.40	1.57	3.48
6-14-56	—	29.0	5.62	10.6
2- 9-57	—	45.0	10.8	—
2-11-57	Bilateral Adrenalectomy (50 mg. cortisone acetate p.o. daily)	—	—	—
2-23-57	—	18.2	2.28	—

* A 56 year old woman who underwent left radical mastectomy for carcinoma of the breast in October 1955 and subsequently received X-ray therapy. Because of hirsutism, obesity with purplish striae, post-menopausal vaginal bleeding, and the palpation of an ovarian mass, she was explored on 2-21-56 and a bilateral oophorectomy performed after finding an ovarian tumor (thecoma). Bilateral adrenalectomy was done on 2-11-57 because of recurrence of the carcinoma of the breast.

mal by adult standards, whereas the β-H.S. excretion was at the lower limit of normal.

Table 5 gives detailed steroid excretion values in a woman with carcinoma of the breast and an ovarian tumor pathologically diagnosed as a thecoma. This woman excreted normal amounts of α- and β-H.S. as well as 17-KS. Following bilateral oophorectomy there was a moderate reduction in 17-KS. and a marked reduction to about one quarter of the pre-operative values of both α- and β-H.S. This decline, however, did not persist and in 4 months the values had risen again to their original levels and in a period of a year the steroid excretion was even higher than pre-operatively. The patient was then subjected to bilateral adrenalectomy because of a recurrence of her carcinoma and following this both the α- and β-H.S. excretion fell. In particular, the β-H.S. fraction decreased to one quarter of its pre-operative value.

Assay of Urinary Steroids Separated by Paper Chromatography. The application of the enzymatic assays to some of the most abundant individual urinary steroids was made possible through the kindness of Drs. T. F. Gallagher and A. Kappas of the Sloan-Kettering Institute, New York, who supplied us with paper strips from their elaborate systems of paper chromatography of urine extracts. The details of preparation of the urine extracts and the separation of the α-ketonic fraction have been described by Kappas and Gallagher (18), and in an earlier paper by Dobriner, Lieberman and Rhoads (19). The urine was subjected to hydrolysis of glucuronides with β-glucuronidase at pH 5, followed by acidification to pH 1 and continuous extraction with ether. After washing with 2N sodium hydroxide and 5% sodium chloride, the ether extract was treated with the Girard T reagent and finally the β-H.S. were separated by digitonin precipitation. Known aliquots of the remaining α-ketosteroids were subjected to paper chromatography in three systems of the type described by Zaffaroni and coworkers (20). The stationary phase consisted of equal volumes of propylene glycol and methanol and all mobile phases were saturated with stationary phases. An α-ketosteroid extract was prepared from the urine of a normal man (age 25) in the manner described by Kappas and Gallagher (18), and aliquots of this subjected to descending paper chromatography on washed Whatman No. 1 paper strips measuring 50×2 cm. The systems employed were: A) A 3.6% aliquot of the 24-hour output was chromatographed for 40 hours with cyclohexane as the mobile phase. Standards of androsterone and etiocholan-3α-ol-17-one were run in parallel. B) A 6.0% aliquot of the 24-hour output was chromatographed for 32 hours with a mobile phase consisting of equal volumes of cyclohexane and toluene. Standards of etiocholan-3α-ol-11,17-dione, androsterone and etiocholan-3α-ol-17-one were run in parallel. C) A 7.2% aliquot of the 24-hour output was chromatographed for 64 hours in the same system as used for B). Standards of etiocholan-3α-ol-11,17-dione, androstane-3α,11β-diol-17-one and etiocholane-3α,11β-diol-17-one were run in parallel. The positions of

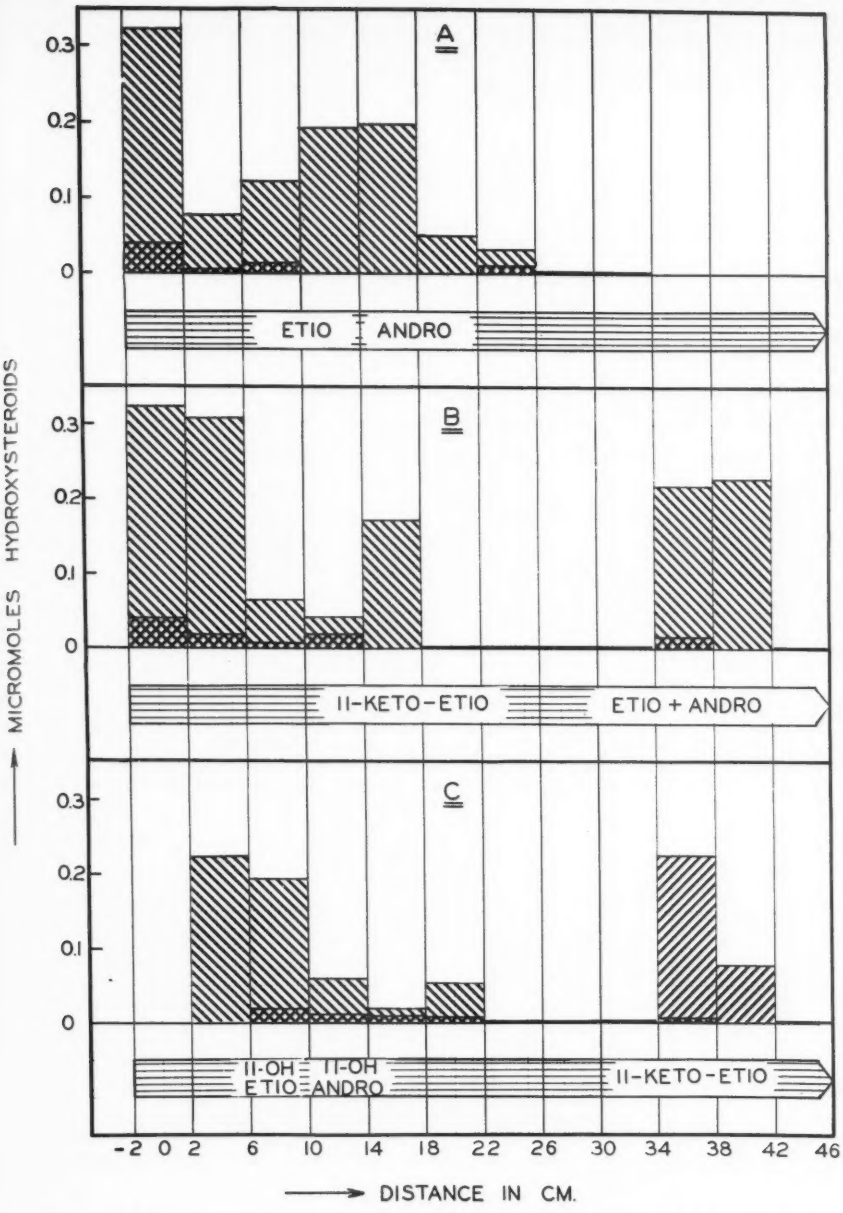

FIG. 5. Enzymatic assays of α- and β-H.S. in segments of paper chromatograms of urine extracts. Three systems A, B and C are shown. Details of the chromatographic systems and amounts of urine extract applied to each strip are given in the text. The extracts were applied at the origin (0 cm.). The strips were cut into 4 cm. segments as indicated by the vertical lines. The amounts of α-H.S. (\\\\\\\) and β-H.S. (XXXX) in each strip are given in μmoles. Clear areas on each paper strip denote the positions of standard compounds as determined by parallel runs. ETIO = etiocholan-3α-ol-17-one; ANDRO = androsterone; 11-KETO-ETIO = etiocholan-3α-ol-11,17-dione; 11-OH-ETIO = etiocholane-3α,11β-diol-17-one; 11-OH-ANDRO = androstane-3α,11β-diol-17-one).

TABLE 6. EXCRETION OF INDIVIDUAL 3α-HYDROXYSTEROIDS DETERMINED BY ENZYMATIC ANALYSIS OF ELUATES FROM PAPER CHROMATOGRAM SEGMENTS

	Calculated from strip*	Micromoles per strip	Micromoles per 24 hr.	Milligrams per 24 hr.
Androsterone	A	0.249	6.95	2.0
Etiocholan-3α-ol-17-one	A	0.318	8.90	2.6
Etiocholan-3α-ol-11,17-dione	B	0.215	3.60	1.1
Etiocholan-3α-ol-11,17-dione	C	0.300	4.16	1.3
Androstane-3α,11β-diol-17-one	C	0.078	1.08	0.33
Etiocholane-3α,17β-diol-17-one	C	0.193	2.68	0.82

* See Figure 5.

standard compounds were indicated on each strip and scale reproductions of these strips are shown in Figures 5A, B and C. The above procedures were kindly carried out at the Sloan-Kettering Institute, New York. The strips were then cut transversely to the direction of solvent travel into 4 cm. segments as shown in Figure 5. Each segment was eluted with 5 to 10 ml. methanol by small scale chromatography. The solvent was evaporated and each residue redissolved in 0.30 to 0.50 ml. CH_3OH, of which 0.10 to 0.15 ml. aliquots were used for enzymatic assay in a 3.0 ml. system with α and β enzymes. The total content of α- and β-H.S. in each paper segment was computed and is represented as a series of bar graphs above each paper strip in Figure 5. Since the aliquot of the 24-hour specimen which was applied to paper is known, calculations of the approximate daily excretion of individual compounds could be made and these are given in Table 6. It can be seen that the digitonin fractionation did not completely separate the α- and β-H.S. and the contamination in some segments of α-H.S. by β-H.S. was 20% or more. The separation of some of the compounds is not complete although this may in part be only apparent because the segments were arbitrarily cut to equal lengths of 4.0 cm. The approximate values obtained for the individual compounds are of the same order of magnitude as those reported by other workers (18, 21). It should be pointed out that no claims are made for the absolute validity of the values here reported. The purpose of these studies is merely to indicate the applicability of the methods to urinary steroids separated by paper chromatography. Confidence in the results given by the enzymatic method depends upon the fact that 3α-H.S. are specifically measured.

DISCUSSION

The reliability and reproducibility of the enzymatic assay procedure for urinary steroids has been investigated in diverse ways, which are summarized here. A strictly linear relation exists between the amount of urine extract and the magnitude of the observed optical density change (Figure 2). This indicates that the reaction equilibria are so adjusted as to favor the quantitative oxidation of hydroxysteroids. Further evidence for this conclusion is derived from the previous demonstration (16) that various pure steroids, singly or in combination, are assayed quantitatively (±1%)

in the presence of an urine extract. Following the addition of steroids of widely varying polarity (androsterone and THE) in physiological amounts to the urine, recovery of these compounds for the entire procedure has been approximately 93% (Table 2). In order to evaluate the reproducibility of the procedure, two samples of a single urine specimen were separately carried through the procedure of preparing an extract and assayed enzymatically. Each sample was assayed with α and β enzymes at three concentrations. The agreement between the duplicates was found to be within 5.0%.

The advantages of the enzymatic estimation of steroids over classical chemical methods have been discussed in detail (11). It may be emphasized that α and β enzymes permit the estimation of the majority of the neutral urinary steroids. The clinical determinations given have illustrated the value of estimating the β-H.S. which have been implicated as indices of abnormalities of function of the adrenal cortex (2). The separation of α-H.S. from β-H.S. by digitonin precipitation has been shown not to be quantitative (Figure 5), and hence cannot be considered reliable.

Acknowledgment

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PATHOLOGIC CHANGES IN ADRENALECTOMIZED AND NON-ADRENALECTOMIZED RATS EXPOSED TO COLD[1]

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ABSTRACT

Sexually mature male rats of the Sprague-Dawley strain were sensitized by removal of one kidney and feeding a diet containing 4 per cent sodium chloride. Adrenalectomized rats were treated with 4 cc. of aqueous adrenal cortical extract per rat per day. Groups of adrenalectomized and non-adrenalectomized rats were each subdivided into groups which were adapted to low temperature and kept at 3–5° C for 60 days, and similar groups kept at room temperature for the same period of time. An additional group of non-adrenalectomized sensitized rats were adapted to the overfeeding of the high salt diet by stomach tube. The amount of fluid diet represented approximately as much dry diet as was eaten *ad libitum* by the cold-exposed rats. The following points were established:

1. The rats exposed to cold ate much more food but gained less weight than did rats kept at room temperature. The rats which were overfed at room temperature became overweight.

2. The adrenalectomized rats treated with adrenal cortical extract at room temperature developed more microscopic damage to the hearts and kidneys than did non-adrenalectomized rats at room temperature, but there was no significant difference in organ weights or in the ratings of gross pathology. It was postulated that the adrenal cortices may modify their secretion of hormones in order to facilitate the excretion of a high salt load. Adrenalectomized rats on a fixed intake of replacement therapy may be at a disadvantage in this situation.

3. When the rats were exposed to cold the gross and microscopic damage to hearts and kidneys were equally marked in adrenalectomized and in non-adrenalectomized rats. There was hypertrophy of hearts and of kidneys in all rats exposed to cold.

4. Rats which were overfed at room temperature showed microscopic damage to hearts and kidneys almost as severe as that seen in the rats exposed to cold.

5. The role of the adrenal glands in causing the pathology which occurred in the cold-exposed rats is uncertain, for two reasons. Food intake was not constant. The amount of adrenal cortical extract used as replacement therapy in the adrenalectomized rats was associated with more damage than occurred in the non-adrenalectomized rats when the comparison was made at room temperature.

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WHEN unilaterally nephrectomized rats are given a high load of sodium chloride and exposed to cold for several weeks, they develop renal and myocardial damage (1, 2). The present experiments were designed to test the hypothesis that such damage is caused by a change in the secretory activity of the adrenal glands. Non-adrenalectomized rats and adrenalectomized rats treated with a constant intake of adrenal cortical extract were studied. When the animals were exposed to cold, damage was caused in both the presence and absence of the adrenals, but the role of the adrenal cortical hormones in causing the damage was not established. Additional evidence was obtained to show that the amount of high sodium chloride diet eaten by the animal is important in determining the extent of damage.

METHODS

Male rats of the Sprague-Dawley strain were maintained on Archer Dog Pellets until the experiment was begun. Unilateral nephrectomy was performed in all rats. During adrenalectomy the glands with as much pedicle as possible were removed without breaking the capsule. Following operation, each rat received 2 cc. of adrenal cortical extract (UPJOHN) in 0.9% saline by subcutaneous injection each morning and afternoon. The sham-operated rats received control injections of saline. The animals were given a special high sodium chloride diet (Table 1). The dry diet was packed tightly into individual Franke animal feeding jars which were weighed every 24 hours. In one experiment the fluid diet was fed by stomach-tube each morning and afternoon. The rats were exposed to cold in a temperature chamber designed by and built under the supervision of the Mechanical Development Division of The Upjohn Company. It was cooled by a sealed refrigeration unit which ran constantly except for automatically controlled defrosting for 15 minutes every 6 hours. The desired temperature was maintained within 1° C by a thermostatic control of heating units built into the air ducts of the machine. The animals were housed in 12 individual metabolism cages contained within the temperature chamber. Twelve similar cages were used outside the chamber for the maintenance of control rats at room temperature (25° C). The initial temperature of the chamber was 25° C. This was lowered 2 degrees each day for 5 days and then 1 degree every second day until the desired low temperature was reached and then kept constant throughout the remainder of the experiment. All of the hair was shaved from each cold-exposed rat twice each week.

TABLE 1. HIGH SALT MEDIUM CARBOHYDRATE DIET

Constituent	Grams
Cellu flour	60
Salt mixture U.S.P. XIV	40
Dried yeast (Pabst)	100
Wheat germ oil	10
Cod liver oil	10
Mazola oil plus 100 mgm. K	10
Mazola oil	190
Casein	160
Starch	300
Dextrin	150
Sucrose	140
Sodium chloride	50
To make fluid diet add water	1040

At the end of the experimental period each rat was anesthetized with ether and exsanguinated, and necropsy was performed. Gross pathology was noted, and the heart, kidney, adrenal glands and thymus were weighed and fixed in Bouin's fluid. One adrenal from each animal was fixed in 10% neutral formalin. Sections of the hearts and kidneys were stained routinely with haematoxylin and eosin and Masson stain. Selected examples were examined by the periodic acid-Schiff routine, Mallory's method, and Weigert's resorcin-fuchsin stain for elastic tisuse. The adrenals were stained with Regand's iron haematoxylin and Masson stain and frozen sections of the formalin fixed glands were examined with Sudan black B and Sudan IV for lipid.

EXPERIMENTS AND RESULTS

In Experiment 1, 24 unilaterally nephrectomized rats ate the dry high-salt diet *ad libitum*. Twelve of the rats were adapted to low temperature and kept at 3–5° C for 60 days, and 12 similar rats were kept at room temperature for the same period of time. These two groups were each divided equally into adrenalectomized and non-adrenalectomized rats. The adrenalectomized animals were each treated with 4 cc. daily of adrenal cortical extract in 0.9% saline, and the non-adrenalectomized rats were each given equal amounts of of 0.9% saline.

Experiment 2 was a repetition of Experiment 1.

In Experiment 3, 8 unilaterally nephrectomized rats were adapted to the over-feeding of a fluid form of the high sodium chloride diet. The food intake was increased 1 cc. daily until the level of 26 cc. per feeding was reached and then kept constant for 60 days. This amount of fluid diet represented approximately as much dry diet as was eaten ad libitum by the cold-exposed rats.

The data are summarized in Tables 2 and 3. The rats exposed to cold ate more food but gained much less weight than did the rats kept at room temperature. The rats which were over-fed at room temperature became overweight.

Heart: There was no significant difference in the average weights of the hearts of control and adrenalectomized rats at room temperature (Table 2). The average weight of the heart was increased to approximately the same extent in the control and adrenalectomized rats exposed to cold. Overfeed-

TABLE 2. BODY AND ORGAN WEIGHTS OF UNILATERALLY NEPHRECTOMIZED ADULT MALE RATS EATING 4% NACL MEDIUM CARBOHYDRATE DIET *ad libitum*. AVERAGES AND STANDARD ERRORS

Experimental conditions	Gms. day food	T° C	No. of rats	Gms. body-weight		Organ weights, mgms.			
				Initial	Final	Heart	Kidney	Thymus	Adrenal
Adrenal glands intact—not shaved	15.2 ±0.32	25	12	339 ±9.1	471 ± 8.9	1120 ± 85.2	1926 ± 56.0	301 ±39.5	25.5 ±0.8
Adrenalectomy, 4 cc. ACE daily—not shaved	14.1 ±0.22	25	12	329 ±8.5	427 ± 5.6	1198 ±100.8	1968 ± 82.9	260 ±21.1	
Adrenal glands intact, shaved twice weekly	29.3 ±0.64	3	10	324 ±6.9	335 ± 9.1	1712 ± 93.0	2704 ±193.4	201 ±31.9	32.0 ±1.5
Adrenalectomy, 4 cc. ACE daily, shaved twice weekly	26.6 ±0.87	3	10	332 ±8.5	340 ± 8.2	1650 ± 66.3	2649 ±183.9	235 ±29.5	
Adrenal glands intact, not shaved; overfed fluid high sodium chloride diet, 52 cc. daily by stomach tube	31.0	25	8	330 ±7.1	660 ±31.8	1576 ±100.8	2669 ±181.0	49 ±21.9	38.0 ±3.5

TABLE 3. RATING OF PATHOLOGY, MEAN STANDARD ERROR AND RANGE

Experimental conditions	T° C	No. of rats	Kidney, gross	Kidney, micro.	Heart, micro.
Adrenal glands intact—not shaved	25	12	0.6 ±0.14 (0 –2.0)	1.2 ±0.29 (0.5–3.0)	0.2±0.16 (0–2.0)
Adrenalectomy, 4 cc. ACE daily—not shaved	25	12	0.75±0.16 (0 –1.5)	2.37±0.39 (0.5–4.0)	1.4±0.26 (0–4.0)
Adrenal glands intact—shaved	3	10	2.6 ±0.46 (1.0–5.0)	3.65±0.43 (0.5–5.0)	1.6±0.24 (0–3.0)
Adrenalectomy, 4 cc. ACE daily—shaved	3	10	3.0 ±0.45 (0.5–5.0)	3.5 ±0.63 (0.5–5.0)	1.5±0.24 (0–4.0)
Adrenal glands intact, not shaved; overfed fluid high NaCl diet, 52 daily by stomach tube.	25	8		2.78±0.55 (0.5–4.0)	1.1±0.31 (0–3.0)

* The ratings of gross renal pathology are based on the following scale: 0 = no abnormality; 1 = few tiny nodules; 2 = moderately nodular with some mottling; 3 = more nodules, mottling with some grey spots; 4 = rough, mottled, with grey and red spots; 5 = changes listed previously more severe giving "flea-bitten" greyish-yellow appearance.

The ratings of the microscopic changes are based upon the following features. *Kidney*: 0 = no abnormality; 1 = hypertrophy of glomeruli and tubules, a few hyaline casts, and slight focal interstitial inflammation; 2 = active pelvic inflammation; 3 = glomerular lesions, no apparent vascular damage; 4 = hyalinization of glomeruli, sclerosis of arterioles; 5 = necrosis of glomeruli and arterioles. *Heart*: 0 = no abnormality; 1 = hypertrophy of muscle; 2 = focal inflammation or fibrosis of myocardium; 3 = changes listed previously, sclerosis of vessel walls; 4 = necrosis of myocardium and/or vessel walls.

ing also caused a gain in heart size of non-adrenalectomized rat kepts at room temperature but the gain was roughly proportional to the gain in body weight. One non-adrenalectomized rat exposed to cold had white patches on the heart, otherwise no gross damage was seen in any heart.

Microscopically the non-adrenalectomized rats maintained at room temperature showed little or no heart damage. All other groups, including force-fed rats, showed a variety of cardiac lesions (Table 3). The most common finding was hypertrophy of the cardiac muscle cells. In many cases this was accompanied by an increase in Anitschkow myocytes, especially around the adventitia of blood vessels and at the auriculo-ventricular junction. Inflammatory lesions took the form either of focal nodules or more diffuse collection of cells. The latter were situated mainly in the subpericardium or subendocardium (Fig. 1). The cells were predominantly of the macrophage and lymphocyte series; polymorphonuclear leucocytes were scanty. The focal lesions did not show the classical appearance of Aschoff nodules.

Areas of fibrosis were situated chiefly near the apex of the heart. They contained a few macrophages laden with hemosiderin pigment. In several instances there was a clear association between myocardial fibrosis and proliferative thickening of small arteries of the coronary system (Fig. 2). Necrosis of arteriolar walls was seen occasionally. The largest coronary arteries appeared normal, apart from one adrenalectomized rat exposed to cold in which there was an eccentric thickening of the arterial wall due to a subintimal collection of foamy macrophages (Fig. 3). This change was suggestive of an early atheromatous plaque. Valvulitis was not seen. In 14

FIG. 1. Heart. Adrenx T 25°. A collection of cells, predominantly of macrophage type, is present in myocardium. Haematoxylin and eosin. ×680.

FIG. 2. Heart. Non-adrenx T 3°. There is replacement fibrosis of the myocardium and arteriolosclerosis of a small arteriole. Masson. ×370.

FIG. 3. Coronary artery. Adrenx T 3°. There is eccentric intimal thickening caused by foamy cells lying superficial to the internal elastic lamina. Weigert's resorcin-fuchsin and Van Gieson. ×680.

rats, however, foci of cartilagenous metaplasia were present at the base at the aorta at the origin of the aortic valve (Fig. 4). Examples of this lesion occurred in all experimental groups and it cannot be said to represent a response to cold-stress.

Kidney: The changes in weights of kidneys paralleled the changes in weights of hearts (Table 2). Exposure to cold and overfeeding caused striking gains in kidney weights but adrenalectomy and treatment with adrenal cortical extract did not.

The kidneys of the non-adrenalectomized rats which ate *ad libitum* at room temperature showed some tiny pits and nodules. The gross damage was somewhat greater for some of the adrenalectomized animals; slight mottling and a few gray spots were noted. The over-fed rats of experiment 3 showed a moderate amount of gross pathology. Gross changes were most severe in adrenalectomized and nonadrenalectomized rats exposed to cold; pits, nodules, gray and hemorrhagic spots were observed. Some of the kidneys appeared pale, very rough and "flea-bitten."

As shown in Table 3, the microscopic assessment of renal damage paralleled the degree of gross damage with the exception of the adrenalectomized animals maintained at room temperature. The severity of damage also varied in animals within a group as noted in the table.

In some rats of all experimental groups mild hypertrophy of nephrons was commonly associated with a few hyaline casts in dilated tubules and small foci of lymphocytes as well as some plasma cells in the interstitial tissue. Near such foci periglomerular fibrosis was seen in some cases. These changes were regarded as minimal kidney damage and probably represented a response to mild infection. In 9 rats there was active chronic inflammation of the renal pelvis. This showed no predilection for any one experimental group but was not seen in any force-fed rat. Furthermore, pelvic infection appeared to be independent of the glomerular and vascular pathology noted in the more severely damaged kidneys.

Glomerular lesions occurred in individual animals of all experimental groups but were minimal in non-adrenalectomized rats at room temperature (Table 3). Tuft damage varied from a slight increase in intercapillary material with adhesion to Bowman's capsule to complete glomerular hyalinization. In some rats the milder type of damage was not associated with any qualitative change in the arterial or arteriolar walls as demonstrated by our histological methods. With more severe damage, however, there was a clear correlation between glomerular lesions and proliferative arteriolosclerosis of the afferent arterioles (Fig. 5). This type of lesion was seen in cold-exposed rats, adrenalectomized rats maintained at room temperature, and also in animals force-fed the high salt diet. The most severe kidney damage was noted in rats exposed to cold, both in the adrenalectomized and non-adrenalectomized groups. Necrosis of the glomerular tuft with thrombosis of some capillary loops was associated with arteriolone-

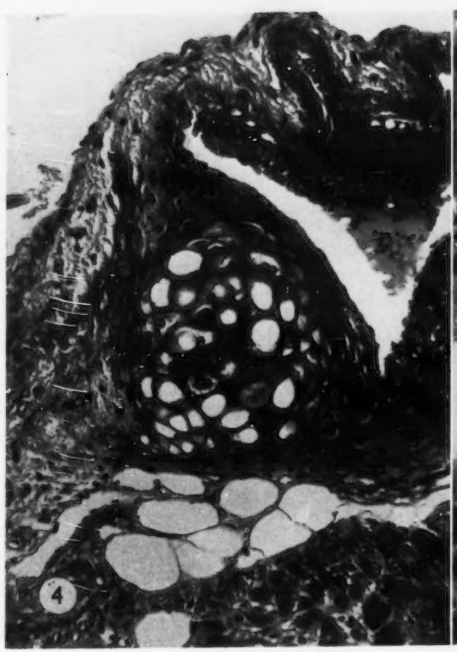

FIG. 4. Aortic valve. Non-adrenx T 25°. An islet of cartilagenous metaplasia is present at the base of the aortic valve. Masson. ×345.

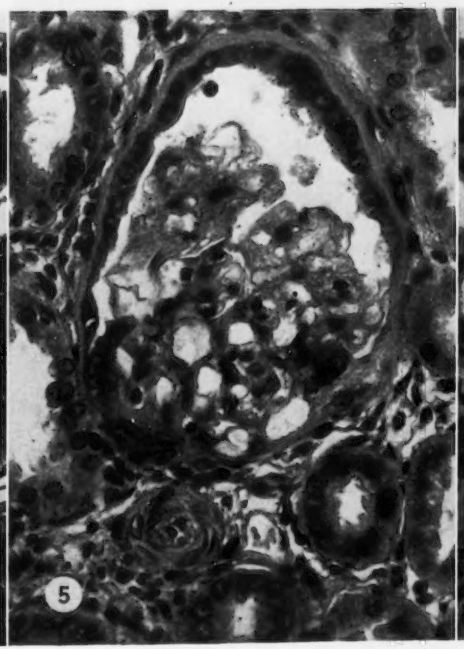

FIG. 5. Kidney. Force-fed T 25°. The glomerular tuft is shrunken and partially adherent to a thickened Bowman's capsule. The parietal epithelium of the capsule is heightened and shows mitotic activity. There is proliferative arteriolosclerosis. H and E. ×760.

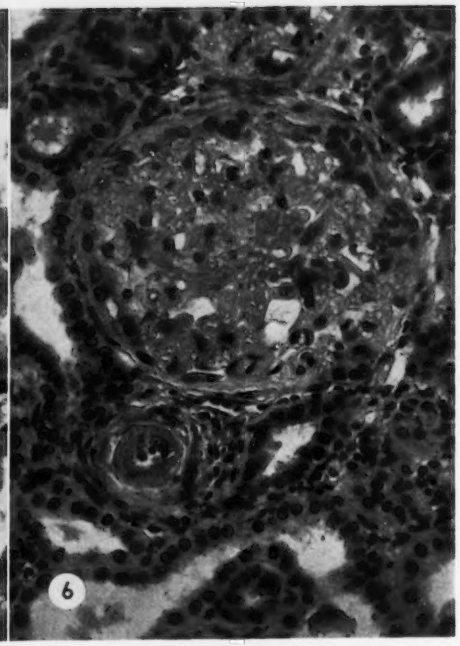

FIG. 6. Kidney. Non-adrenx T 3°. The glomerulus is enlarged and shows patchy necrosis; arteriolonecrosis of the afferent arteriole. H and E. ×500.

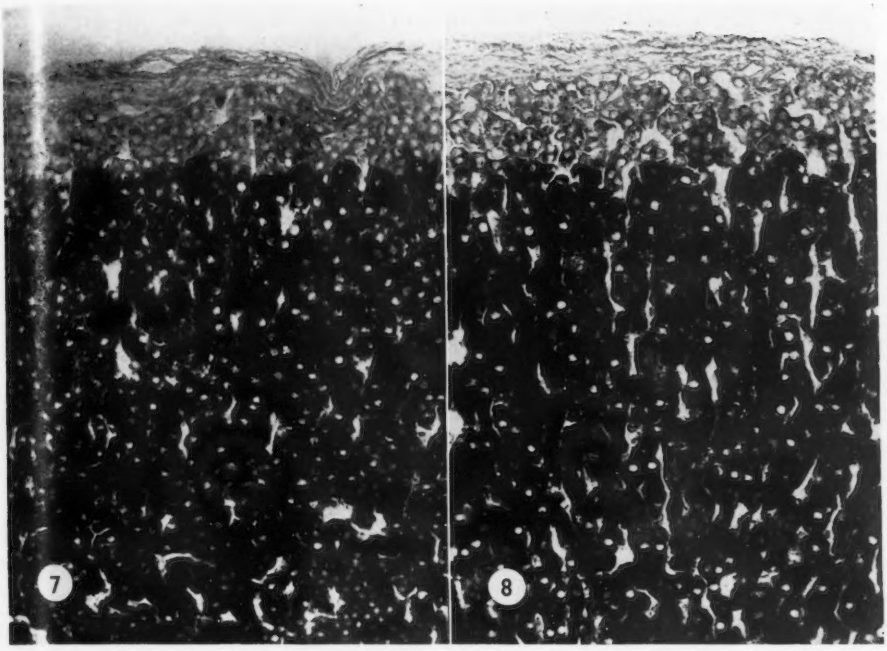

FIG. 7. Adrenal. Non-adrenx T 25°. Zona glomerulosa is narrow and contains almost no lipid. Frozen section. Sudan black. ×380

FIG. 8. Adrenal. Non-adrenx T 3°. Zona glomerulosa is narrowed to the same extent as Fig. 7 and shows similar lipid depletion. Frozen section. Sudan black. ×380.

crosis of the afferent vessel (Fig. 6). In one cold-exposed adrenalectomized rat this necrotic change also involved small arteries.

Adrenals: The adrenals from cold-exposed rats were slightly larger than the controls at room temperature. Mild adrenal hypertrophy occurred in force-fed rats (Table 2).

The zona glomerulosa in rats maintained in the cold and at room temperature was narrow. It was composed of small cells in which the cytoplasm was dense and uniform. Lipid in the glomerulosa of both groups was almost completely depleted, apart from a few granules associated with the Golgi apparatus (Fig. 7 and 8). An increase in breadth of the zona fasciculata and zona reticularis was seen in the cold-exposed rats. Lipid staining was more abundant and the cells of this zone were slightly larger than the room temperature controls.

DISCUSSION

These experiments confirm earlier observations (1, 2) that exposure of the sensitized rat to cold is associated with increased damage to the heart and to the remaining kidney and that the increase in food-intake is an im-

portant factor determining the extent of damage. Sensitized rats kept at room temperature and tube-fed approximately the same amounts of diet eaten by the cold-exposed rats had more damage than did rats eating *ad libitum* at room temperature. However, damage was greatest in the rats exposed to cold. In an earlier study (2) it was shown that damage among cold-exposed rats is less when the intake of the high-salt diet is restricted to a normal level, although it remains somewhat greater than among rats kept at room temperature. It seems probable that cold-exposure increases damage when the intake of the high-salt diet is constant at either normal or high levels, but that when the dietary load does change the extent of variation is important.

Treatment of the adrenalectomized-sensitized rat with adrenal cortical extract at a level of 4 cc. per rat per day apparently places the animal at a disadvantage in dealing with a high salt load as compared to similar rats having the adrenal cortices intact. The adrenalectomized rats given substitution therapy and kept at room temperature had more microscopic damage to the hearts and kidneys than non-adrenalectomized rats. These differences were not evident grossly and were not reflected by differences in organ weights. Ingle and Baker (3) have reported on the damaging effects of adrenal cortical extract in the sensitized rat. These results are interesting and important, for we have considered this level of replacement therapy to be a "normalizing" dose of hormones. Indeed, smaller doses of adrenal cortical extract do not suffice to maintain life in adrenalectomized cold-exposed rats, at least during the early stages of adaptation to cold. Perhaps the rat having intact adrenal glands is able to suppress the secretion of sodium-retaining hormones when the animal is exposed to a high sodium chloride load. Or, possibly the adrenal cortices can increase the secretion of hormones which facilitate the excretion of sodium and chloride. The observation that the zona glomerulosa was narrow, lipid depleted and composed of smaller than normal cells may be indirect evidence that the adrenal cortices undergo changes in secretory activity which favor the maintenance of homeostasis of electrolytes. These considerations are consistent with the observations and concepts of Deane, Shaw and Greep (4) relative to the role of the zona glomerulosa in the secretion of sodium-retaining steroids.

Our objective in these and in related studies has been to design experiments having only one variable under study at a time. We have not yet succeeded. Food intake has remained inconstant. When rats are exposed to cold they must eat more food or they will die. We have tested the possibility of force-feeding all of the rats at the level consumed voluntarily by the cold-exposed rats. When either adrenalectomized or non-adrenalectomized rats are force-fed they will die when exposed to cold. The stressed rats cannot handle a high salt load imposed suddenly twice daily, whereas they will tolerate the same total amounts when consumed gradually

throughout the day. The information presently available to us would not exclude the possibility that adrenalectomized rats at room temperature given adrenal cortical extract and a dietary load equivalent to that eaten by cold-exposed rats would develop as much damage as rats exposed to cold.

When the stress of cold and of the increased dietary load both impinge upon the adrenalectomized-sensitized rat given a steady intake of adrenal cortical extract, it seems probable but not certain that the microscopic damage to the kidney is greater than occurs in similar rats at room temperature. Our uncertainty as to whether a change in the secretory activity of the adrenal cortices mediates any or all of the damaging effects of cold stress is analogous to the position of Ingle and Baker (3) following a study of the role of the adrenal glands in mediating the damaging effect of lyophilized anterior pituitary (LAP). There also, treatment of adrenalectomized-sensitized rats with adrenal cortical extract caused more damage than was seen in non-adrenalectomized sensitized rats; the addition of LAP was judged to aggravate the microscopic damage in the absence of the adrenals but the validity of the difference was not established beyond doubt.

The presence or absence of the adrenal cortical hormones or the level of replacement therapy in adrenalectomized animals is an important part of the pattern of causes determining the amount of pathology under the unusual conditions of these experiments.

Blood pressure records were not taken in our studies. It seems most likely, however, that the cardiac hypertrophy and renal and vascular changes of the more severely affected rats was associated with an elevation of blood pressure. One might speculate that the more intense renal damage (rated 4 and 5) represented a response in the rat comparable to that seen in benign and malignant hypertension in man. The random distribution of overt renal infection bore no close relation to any of our experimental procedures and the incidence of cartilagenous metaplasia at the base of the aorta varied similarly. It seems probable that the latter change is related to the appearances described in the aorta of the ageing rat by Ingle and Baker (3), rather than a result of our experimental design, although the rats used here were young adult males. Sellers and You (5) have reported cardiovascular lesions with demonstrable lipid in the coronary arteries of normal rats fed a normal diet during exposure to cold for 10 to 18 months. Only one of our cold-exposed rats, an adrenalectomized animal, showed changes in a coronary artery suggestive of atheroma.

There is an impressive amount of evidence that the adrenal glands and their hormones are involved in a number of diseases of man. It is important to note in the present studies that there was no evidence of acute rheumatism in the heart, polyarteritis nodosa, arthritis, peptic ulcer, or any other condition which might be considered a "disease of adaptation." Is Selye (6)

correct in supposing that derailment of pituitary-adrenal functions during exposure to stress is the primary cause of these experimentally induced and many naturally occurring diseases? Or, is the role of the adrenal glands in the etiology of disease a permissive (7) one which requires no departure from the normal role of adrenal hormones in the economy of the body? These questions are of basic importance in nosetiology and there is no body of evidence which compels a decision at this time.

Acknowledgment

We wish to express our appreciation to Dr. Robert O. Stafford of The Upjohn Company, who supplied the adrenal cortex extract used in these experiments.

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NOTES AND COMMENTS

PREVENTION OF EXPERIMENTAL NEPHROCALCINOSIS WITH THYROXINE[1]

ABSTRACT

Experiments on rats indicate that the nephrocalcinosis normally produced by an excess of dietary phosphate can be inhibited, both by thyroparathyroidectomy and by treatment with thyroxine.

It has long been known that nephrocalcinosis and renal calculus formation can be produced by an excess of parathyroid hormone, but during the last 15 years it has become increasingly more evident that several other endocrine principles also participate in the production of such lesions. In particular, it could be shown that the nephrocalcinosis produced, in the rat, by an excess of phosphate is aggravated by mineralocorticoids and inhibited by glucocorticoids (1, 2, 3).

Folliculoid, or "estrogenic" hormones exert a pronounced effect on calcium metabolism in birds, presumably because in the avian species the production of calcified egg shells is closely related to the action of sex hormones. In mammals, on the other hand, folliculoids do not exert any comparably pronounced effect on calcium metabolism, under ordinary conditions. It was of interest to note, therefore, that in rats given threshold doses of NaH_2PO_4, estradiol (like mineralocorticoids) induces pronounced nephrocalcinosis (4).

These findings suggested that the rat sensitized by oral administration of an excess of phosphates represents a highly suitable test object for the study of hormonal factors capable of influencing renal calcification. It is the object of this communication to record experiments which show that the thyroid hormone likewise exerts an important influence upon the production of this kind of nephrocalcinosis.

MATERIALS AND METHODS

Fifty female Sprague-Dawley rats, with an average initial body-weight of 100 gm. (range: 95–108 gm.), were subdivided into five equal groups, as indicated in Table 1.

The animals of all five groups received a 10% aqueous solution of NaH_2PO_4, at the dose of 2 ml., twice daily, by stomach tube. The concentration of this solution was raised to 15% on the 14th day, without changing the volume of fluid administered daily. *Group I* was not otherwise treated; *Group II* was thyroparathyroidectomized on the first day of the experiment, under ether anesthesia, while *Groups III, IV and V* received sodium thyroxinate in 0.2 ml. of water, subcutaneously, daily, at the dose levels of 25 μg., 250 μg., and 500 μg., respectively.

The degree of nephrocalcinosis was estimated both macroscopically (judged by the appearance of whitish spots on the outer and cut surface of the kidneys), and microscopically (after staining neutral-formalin-fixed, paraffin-embedded sections with Kossa's silver nitrate and the celestine blue[2] techniques). The intensity of calcification was gauged in terms of an arbitrary scale of 0 to +++. In addition, the kidneys were weighed on an analytic balance, because intense nephrocalcinosis is usually accompanied by an increase in renal weight.

The experiment was terminated after 20 days, and the principal data are listed (with their standard errors, wherever indicated) in Table 1.

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[2] 20 minutes in 0.1 aqueous solution of "Celestine Blue B" (British Drug Houses), to which is added 1 ml. of 1% ammonia water; 2) rinse briefly in 70% alcohol to remove excess stain, and counterstain, as desired (e.g., in carmalum or safranin 0).

TABLE 1. PREVENTION OF EXPERIMENTAL NEPHROCALCINOSIS WITH THYROXINE

Group	Treatment*	Final body-weight (gm.)	Renal weight (mg.)	Nephro-calcinosis
I	None	174 ± 4.4	1459 ± 77.3	+++
II	Thyroparathyroidectomy	123 ± 6.5	620 ± 32.9	0
III	Thyroxine 25 μg/day	163 ± 4.3	1349 ± 51.9	0 to +
IV	Thyroxine 250 μg/day	148 ± 5.4	1303 ± 38.0	0
V	Thyroxine 500 μg/day	143 ± 2.9	1312 ± 36.0	0

* In addition to the treatments indicated in this column, all animals received NaH_2PO_4 by stomach tube, as outlined in text.

RESULTS

It is evident from our findings that both thyroparathyroidectomy and thyroxine prevented the nephrocalcinosis normally produced by the administration of excess phosphate. In the case of thyroparathyroidectomy, this inhibition of calcification was accompanied by a pronounced decrease in renal weight (such as is characteristic of thyroid deficiency), but the kidneys of the thyroxine-treated rats were not markedly smaller than those of the controls.

Histologic examination of the kidneys (Fig. 1) revealed numerous calcified renal casts (particularly at the cortico-medullary junction) in all of the control rats, and a few casts in some of the rats which had received the smallest dose of thyroxine (25 μg./day). Such calcium deposits were not visible, either in the thyroparathyroidectomized animals or in those treated with the higher doses of thyroxine (Groups IV and V).

FIG. 1.—*Left:* Extensive calcification at the cortico-medullary junction line in the kidney of a rat of Group I, treated with NaH_2PO_4 alone.—*Right:* Complete absence of calcification depositions in the kidney of a rat of Group IV which, in addition to the NaH_2PO_4, was also given 250 μg./day of thyroxine (×10.5).

In soft tissues other than the kidney, no calcium deposition was seen in any group.

No signs of tetany were detectable in the thyroparathyroidectomized rats, but a relative parathyroid deficiency may have participated in the inhibition of nephrocalcinosis in this group. This point will be checked on parathyroidectomized rats in another investigation designed to explore the possible participation of parathyroid hormone in this type of nephrocalcinosis. However, the findings reported here clearly indicate that comparatively small doses of thyroxine suffice to protect the kidney against the production of nephrocalcinosis by an excess of dietary phosphate.

These experiments furnish an additional instance demonstrating the important "conditioning" role of hormones in the production of disease susceptibility.

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CORTICOSTEROID FORMATION IN REGENERATING RAT ADRENALS[1]

ABSTRACT

Adrenal regeneration in uninephrectomized, uniadrenalectomized young rats on a salt diet is accompanied by hypertension, which may be due to abnormal adrenal secretions. To test this hypothesis, chemical analyses were performed on the 21st and 30th day after adrenal enucleation. Corticosteroids were measured by the blue tetrazolium reaction and by paper chromatography in the adrenal vein blood and in the medium in which the glands were incubated. The results indicate that during adrenal regeneration there are no excessive amounts of aldosterone nor any significant imbalance in the steroid mixture, as compared with normal adrenals; this applies regardless of the presence of ACTH.

Adrenal enucleation with contralateral adrenalectomy under appropriate conditions is followed by adrenal regeneration and hypertension (1). In the rat, the associated factors namely uninephrectomy, salt excess and youth are similar to those sensitizing to desoxycorticosterone hypertension (2, 3). The regenerating adrenal tissue takes origin from the cells of the zona glomerulosa left attached to the capsule. Aldosterone is of glomerulosal origin (4) and is known to provoke clinical hypertension when secreted in excess (5), so that adrenal regeneration hypertension could be attributed to an ab-

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solute or relative excess of this hormone. The purpose of the present investigation was to test these concepts on the pathogenesis of adrenal regeneration hypertension. Two series of experiments were done. In these, steroid formation by incubated regenerating rat adrenals was studied on the 21st and 30th postoperative days respectively.

METHODS

200 Sprague-Dawley female rats weighing 54 to 90 gm. were operated and maintained as indicated by Skelton (1). With each series 30 control rats were uninephrectomized and homolaterally uniadrenalectomized and given 1% NaCl to drink. Since a variable percentage of rats with regenerating adrenals develops hypertension (6), blood pressure was taken once a week during the first 14 days, and twice weekly thereafter. This enabled us to establish with certainty the presence of hypertension (systolic pressure greater than 150 mm. Hg) in the animals subsequently to be tested for steroid formation.

We had no indication of the most appropriate period during which the biochemical studies should be conducted, and decided arbitrarily on the 21st day, by which the onset of hypertension is usually demonstrable, and the 30th day, when hypertension is already established in those animals developing the syndrome. Towards the end of each experiment the experimental rats and their controls were sent to the laboratories of the Worcester Foundation for biochemical studies.

The adrenal glands were removed under pentobarbital anesthesia, quartered and incubated in a Dubnoff incubator as described by Saffran and Schally (7) with some modifications. Thus in experiments wherein the response to adrenocorticotropic hormone (ACTH) was investigated, each flask contained adrenal tissue equivalent to one gland; in experiments wherein the corticoids released during the incubation period were isolated by paper chromatography, each flask contained tissue equivalent to 2 glands. The incubation medium was Krebs-Ringer-bicarbonate-glucose (200 mg.%) and the final volume in each flask 2.0 ml. After an incubation period of 60 minutes under 95% O_2 and 5% CO_2, the incubation medium was extracted with methylene dichloride and the extracted corticoids assayed colorimetrically by the blue tetrazolium (BT) reaction as outlined by Elliott *et al.* (8). Evidence for the identity of the corticosterone isolated (compd B) on paper chromatograms run in the toluene-propylene glycol system (TPG) (9) has been presented elsewhere (10). The material isolated from the TPG chromatogram run-offs run in the ligroin-propylene glycol system (11) is presumably desoxycorticosterone (DOC) by virtue of its mobility when compared with standard DOC. ACTH solutions were made up fresh in physiological saline and added to the incubation medium in a volume of 0.1 ml. prior to the final incubation. Two hundred sixteen milliunits (mU) were added per flask unless otherwise designated. ACTHar-ACTH, Blend 2-H was obtained from the Armour Laboratories; Bell ACTH peptides (12) from Dr. Paul H. Bell of the Lederle Laboratories and the Wilson corticotropin, pure, from Dr. C. E. Graham of the Wilson Laboratories.

RESULTS

I—*Adrenals tested on the 21st day*

Table 1 indicates that the regenerating adrenals respond to ACTH. However the amount of reducing material formed by these glands is less in the experimental than in the control rats. The fact that responses were obtained from all three ACTH preparations shows that the responses to the relatively crude ACTHar is due to its ACTH content and not to contaminants.

The amounts of steroids isolated on paper chromatograms from the incubation media are shown in Table 2. The steroid content of a small aliquot of the methylene dichloride extract of the incubation medium prior to paper chromatography is given in Column A. The latter were obtained from large batches of adrenals from control and

TABLE 1. RELEASE OF BLUE TETRAZOLIUM (BT) REDUCING MATERIAL (IN μG./100 MG. OF TISSUE) BY INCUBATED RAT ADRENALS

Groups	No ACTH	ACTHar ACTH, 300 mU.	Bell B ACTH, 250 mU.	Wilson Pure ACTH, 72 mU.
Control—8 glands	12.41	38.10	31.16	31.55
Experimental—8 glands	4.79	17.68	11.58	10.65

TABLE 2. AMOUNT (IN μG./100 MG. OF TISSUE) OF COMPOUND B AND DOC ISOLATED ON PAPER CHROMATOGRAMS

Group and treatment	Weight of tissue, mg.	A Steroid found before paper chromatography*	B Compound B	C DOC	D Total compound B+DOC
Control					
— 22 glands	614	9.78	9.30	None	9.30
ACTH 24 glands	651	35.90	20.90	0.96	21.86
Experimental					
— 31 glands	693	12.10	4.86	None	4.86
ACTH 43 glands	940	17.20	11.42	0.57	11.99

* Values in this column were obtained from the analysis of an aliquot of the pooled methylene dichloride extract prior to paper chromatography.

hypertensive animals following incubation with and without ACTH. It can be seen that the compound B+DOC values are similar to those which were found by direct analysis of the methylene dichloride extract, with the exception of that given in Column A for the experimental adrenals which were incubated without ACTH (12.10 μg.). This high value, which is presumably due to some error in the assay, could not be checked since all extracts were subsequently used for paper chromatography. The fact that the figure of 4.86 μg. obtained for compound B (Column B) agrees with the corresponding value found in Table 1 (4.79 μg.) justifies this assumption.

We would like to emphasize that the adrenals of both control and experimental animals produced less aldosterone, cortisol and 6β-OH DOC migrating material on chromatograms than what is usually found in non-uninephrectomized, non-uniadrenalectomized rats. From past experience, similar tests in normal rats gave an aldosterone spot which reacted with BT on paper. We have no definite explanation of this difference.

II—*Adrenals tested on the 30th day*

In this experiment the adrenals from the experimental rats were divided into 2 batches according to the degree of hypertension: one batch from rats with blood pressure between 150 and 190 mm. Hg and the other batch from rats with pressure greater than 190 mm. Hg.

As can be seen in Table 3, there is no marked difference in the liberation of adrenocorticosteroids between the control and the experimental animals when the adrenals are not stimulated by ACTH. Glands from both groups responded to ACTH; the highest yield (37.20 μg.), which was observed in the controls, corresponds to what can be expected from animals with both adrenals.

TABLE 3. RELEASE OF BLUE TETRAZOLIUM (BT) REDUCING MATERIAL (IN μG./100 MG. OF TISSUE) BY INCUBATED RAT ADRENALS

Group	No ACTH	ACTH—217 mU.
Control 4 glands	8.25	37.20
Experimental Mild Hypertension 4 glands	8.87	23.10
Experimental Severe Hypertension 4 glands	8.48	24.80

TABLE 4. AMOUNT (IN μG./100 MG. OF TISSUE) OF COMPOUND B AND DOC ISOLATED ON PAPER CHROMATOGRAMS

Group and treatment	Weight of tissue, mg.	A Theoretical BT values	B Compound B	C DOC	D Compound B+DOC
Control					
— 10 glands	275	8.25	6.55	0.81	7.36
ACTH 12 glands	329	37.2	26.3	2.22	28.5
Experimental					
— 14 glands	319	8.68	9.90	1.45	11.35
ACTH 16 glands	359	23.95	12.5	1.39	13.89

Since the production of corticosteroids with or without ACTH was found to be the same in the mild and in the severely hypertensive rats, no differentiation was made in choosing the animals for the large scale incubation. The results from paper chromatography are indicated in Table 4. The values obtained for corticosterone and desoxycorticosterone agree with those calculated on the basis of the results listed in Table 3. As in the glands tested, at the 21st day, control and experimental animals produced insignificant amounts of aldosterone.

COMMENT

Two concepts of adrenal malfunction, which might underlie the hypertension occurring during adrenal regeneration, were formulated. The first postulated an increased secretion, notably of aldosterone and the second the possibility of an imbalance of the steroid mixture formed with relative aldosterone excess. The present experiments do not support either hypothesis. As a matter of fact, the amounts of aldosterone released by incubation were smaller than those found in similar tests using adrenals from normal (non-uniadrenalectomized) rats. We do not know whether this difference is due to the strain of rats, to uniadrenalectomy or to the administration of salt. There remain the possibilities that steroid imbalance or aldosterone excess may have been present before the onset of hypertension and may have predisposed thereto, and also the possibility that some unknown steroid with pressor activity may be formed. In the latter case, this unknown pressor steroid would be represented by the difference in the ACTH experimental animals between the theoretical and the found BT material, assuming that this difference were significant.

However, Grollman (13), from purely biological experiments, has concluded as we have, that there is no noteworthy adrenal dysfunction during the period of adrenal regeneration. We are, therefore, led to suggest that the hypertension of adrenal regeneration represents a sensitization of the vascular tree to the normal hormones formed by the regenerating adrenal. It is assumed that this sensitization occurs during the initial period of adrenal hypofunction, which follows on adrenal enucleation, and is equivalent to the pressor hypersensitivity of patients with Addison's disease with regard to desoxycorticosterone. On this basis one could explain the hypersensitivity to renin of rats with adrenal enucleation (14) and also the antihypertensive activity of amphenone in such animals (15).

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ADDENDUM

Samples of adrenal vein blood from control unilateral adrenalectomized rats and from rats with regenerated adrenals have been analyzed for steroids.[2] Chloroform extracts of whole blood were partitioned between 70% methanol and pentane. The methanol fraction was chromatographed on a propylene glycol-toluene system. Acetates of the various fractions were prepared and chromatographed on a full strength propylene glycol-hexane system, identified and quantitated. Corticosterone was estimated to be secreted at a rate of 0.52 μg./min. in the control animals and at a rate of 0.24 μg./min. in the animals with regenerating adrenals. The concentration of corticosterone in the adrenal vein blood of control and experimental animals was 3.7 and 1.3 μg./ml., respectively. An acetate of an unidentified steroid with polarity similar to that of 11-desoxycorticosterone acetate was present in significant quantity in the control sample. This steroid was barely detectable in the adrenal vein blood of the animals with regenerating adrenals. Analysis was limited to corticosterone and the relatively nonpolar substance. The results suggest that the regenerating adrenal secretes corticosterone and an unidentified steroid of low polarity at subnormal rates. The possibility of secretion of a steroid with unusual properties directly related to the hypertension of the experimental animals remains unanswered. The results of this study are, in general, consistent with those reported above.

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[2] The steroid analyses of adrenal vein blood were conducted by K. Ballard, M. Lipscomb and G. Sayers, Department of Physiology, Western Reserve University School of Medicine.

PROGESTINS OF THE BOVINE OVARY

ABSTRACT

Ovaries from non-pregnant cows and heifers, obtained shortly after slaughter, were extracted with ethyl acetate and then defatted in cold 70% methanol.

The resulting extracts were chromatographed on paper and the isolated compounds identified by: (1) mobility on paper, (2) formation of derivatives and (3) ultra-violet and sulfuric acid spectra.

Quantitative estimates from ultra-violet spectra indicated 4.33 μg. of progesterone and 0.79 μg. of Δ^4-pregnene-20β-ol-3-one per gram of ovarian tissue. A trace of an ultra-violet absorbing compound that chromatographed like Δ^4-androstene-3,17-dione or Δ^4-pregnene-20α-ol-3-one and a very polar material were also detected.

The isolation and identification of progestational compounds other than progesterone from the bovine ovary has not been reported previously.

Edgar (1) has reported the isolation of 20 μg. of progesterone in 60 grams of corpora lutea from a gonadotrophin-stimulated calf.

The *in vitro* conversion of progesterone to Δ^4-pregnene-20β-ol-3-one by bovine corpus luteal tissue has been demonstrated by Hayano, *et al.* (2).

Zander, *et al.* (3, 4) have recently demonstrated the presence of both the α and β isomers of Δ^4-pregnene-20-ol-3-one in human ovaries. They reported that the α isomer was present in larger quantities than the β isomer.

This note describes the identification and quantitative estimation of progesterone and Δ^4-pregnene-20β-ol-3-one isolated from bovine ovaries.

Only tissues from cows or heifers that were not pregnant and which had grossly normal reproductive tracts were selected. Ovaries were removed shortly after slaughter and immediately placed in cold ethyl acetate. Within 3 hours after removal, pooled samples of 100 to 150 gm. of ovarian tissue were extracted in a Waring blender with ethyl acetate. After evaporation of the solvent, the residue was taken up in warm 70% methanol. The 70% methanol solution was stored overnight at −5° C and then centrifuged in the cold to remove fat as described by Zander and Simmer (5). The supernatant, after evaporating off most of the methanol, was diluted with water and extracted with ethyl acetate. The ethyl acetate extract was dried over Na_2SO_4 and then evaporated to dryness under a stream of nitrogen.

The resulting residue was then chromatographed on paper for four hours in the hexane-formamide system. Four zones of absorption were detected with ultraviolet light. Zone one showed a chromatographic mobility similar to authentic progesterone. This compound had ultraviolet and concentrated sulfuric acid spectra exactly like authentic progesterone.

Zone two chromatographed like Δ^4-pregnene-20β-ol-3-one. It formed an acetate in acetic anhydride-pyridine (1:1) that chromatographed like the acetate of Δ^4-pregnene-20β-ol-3-one. Zone two was oxidized to progesterone by 1.8% chromic acid in acetic acid. This zone gave ultraviolet and sulfuric acid spectra similar to those of authentic Δ^4-pregnene-20β-ol-3-one.

Zone three chromatographed like Δ^4-pregnene-20α-ol-3-one or Δ^4-androstene-3,17-dione. Insufficient amounts were present for further identification (Table 1).

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TABLE 1. QUANTITATIVE ESTIMATION OF PROGESTINS IN 310 GRAMS OF BOVINE OVARIAN TISSUE

Compound	Weight of tissue	Total steroid	Steroid/gm. ovarian tissue
	gm.	μg.	μg.
Progesterone	310	1344	4.33
Δ^4-pregnene-20β-ol-3-one	310	246	0.79
Zone three	310	<25*	<0.08

* Comparative estimate from chromatogram.

Zone four, found at the origin, is a very polar material and is as yet unidentified.

Table 1 gives quantitative estimates of progesterone and Δ^4-pregnene-20β-ol-3-one found in 310 gm. of ovarian tissue. Estimates were from ultraviolet absorption spectra. It can be seen that progesterone is the principal progestin in the bovine ovary but that a significant amount of Δ^4-pregnene-20β-ol-3-one is also present. The 20α isomer, if present at all, is in quite low amounts.

The role of these 20-hydroxy progestins in the physiology of reproduction should be more thoroughly explored. Isolation of these steroids from ovaries and the relative biological differences in the Clauberg assay as compared to the Hooker-Forbes assay (Zander *et al.* 3, 4) may be indicative of a special function which these steroids play.

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TRAINING PROGRAM FOR STEROID BIOCHEMISTRY

Applications are now being accepted for the third course in the Training Program for Steroid Biochemistry which will begin October 1, 1958. The training program is sponsored by the National Cancer Institute of the National Institutes of Health to provide specialized training for individuals interested in steroid investigations.

The program is conducted by personnel at the Worcester Foundation for Experimental Biology with the Department of Chemistry, Clark University, Worcester, Massachusetts, and the Department of Biochemistry, College of Medicine, University of Utah, Salt Lake City.

Two groups of candidates will be selected for training. Post-doctoral candidates having an M.D. or a Ph.D. degree will receive $5000 for a one year training period extending from October 1, 1958 through September 31, 1959. The course will consist of laboratory instruction and lectures covering the theoretical and practical aspects of steroid research, and a period of research on a steroid problem with an established investigator.

Candidates having a B.S. or M.S. degree or equivalent training will receive $1500 for a six months' period extending from October 1, 1958 through March 31, 1959. The pre-doctoral program is intended to provide competency in the analysis of steroid substances for research and clinical laboratories.

The closing date for applications will be June 1, 1958. Requests for applications should be made to the Training Program for Steroid Biochemistry, Department of Chemistry, Clark University, Worcester, Massachusetts, or to Dr. Kristen Eik-Nes, Department of Biochemistry, College of Medicine, University of Utah, Salt Lake City, Utah.

NATIONAL ACADEMY OF SCIENCES

The Division of Medical Sciences of the National Academy of Sciences—National Research Council is accepting applications for grants-in-aid of research for consideration by the Committee for Research in Problems of Sex. The funds for support of this program are provided by the Rockefeller Foundation and the Ford Foundation. The Committee is concerned primarily with encouraging research on the mechanisms underlying sexual behavior, with special emphasis on the higher mammals and man. Proposals involving endocrinological, neurological, psychological, anthropological, phylogenetic, and genetic studies directed toward this objective are therefore invited. Requests that deal with the physiology of reproduction or with related biological and biochemical fields should be addressed to the Committee only if they give promise of shedding light upon behavioral mechanisms.

Preliminary inquiries should be addressed to Room 309, Division of Medical Sciences, National Academy of Sciences—National Research Council, 2101 Constitution Avenue, N. W., Washington 25, D. C. Completed applications for the fiscal year 1958–1959 should be post-marked on or before March 7, 1958.